INSIGHTS INTO MARINE NITROGEN CYCLING IN COASTAL SEDIMENTS: INPUTS, LOSSES, AND MEASUREMENT TECHNIQUES

A Thesis
Presented to
The Academic Faculty

by

Cynthia Adia Hall

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Earth and Atmospheric Sciences

Georgia Institute of Technology

May, 2009

INSIGHTS INTO MARINE NITROGEN CYCLING IN COASTAL SEDIMENTS: INPUTS, LOSSES, AND MEASUREMENT TECHNIQUES

Professor Ellery Ingall, Advisor
School of Earth & Atmospheric Sciences
Georgia Institute of Technology

Dr. Andrew Stack
School of Earth & Atmospheric Sciences
Georgia Institute of Technology

Dr. Greg Huey
School of Earth & Atmospheric Sciences
Georgia Institute of Technology

Dr. Joseph Montoya
School of Biology
Georgia Institute of Technology

Dr. Judith Curry
School of Earth & Atmospheric Sciences
Georgia Institute of Technology

Date Approved:
September 18, 2008

Acknowledgements

I would like to thank my advisor, Dr. Ellery Ingall, for accepting me into his group and for being a great coach over the years. He has been a good friend and great mentor. I also want to thank Dr. Ashanti Pyrtle for mentoring me in my early years as a grad student and for truly inspiring me to accomplish all of my goals. My friends in EAS, Poulomi, Melanie, Steph Chow, Nunu, Beth, Jud, Anne and Karari, gave me support through laughter and sound advice. I would like to thank my committee members, Dr. Greg Huey, Dr. Judith Curry, Dr. Joseph Montoya, Dr. Andrew Stack, and Dr. Martial Taillefert, for taking the time to guide me through the research, writing, and revising processes. I would like to thank the staff of EAS, particularly Susan Ryan, Kathy Plummer, and Chris Lowell. I would like to thank my family, who made this dream a realization for me. My mother and father gave me unlimited support by helping us with the children and there is no way I would have finished without that help. My brother-in-law, Joemaiah, continued to see me through the last days of my dissertation by looking after the children any time I needed the help. My children, Sitafa James and Adia Sulé, inspired me to finish. Lastly, I would like to thank my husband, Micaiah, for standing by me through this entire ordeal. This last year put such a strain on our family and he never stopped supporting and encouraging me, although I did not always understand the way in which he showed his support. I hope to be able to support him throughout his life the way that he supported me during this time. Most importantly, I would like to thank all of my ancestors who made my life possible. Axé

TABLE OF CONTENTS

List of Tables

List of Figures

SUMMARY

Denitrification mechanisms and rates were determined in Georgia continental shelf and salt marsh sediments. Isotope tracer and membrane inlet mass spectrometry (MIMS) techniques were employed during all denitrification studies in this dissertation. MIMS measurements were also improved by investigating and optimizing experimental parameters.

Canonical heterotrophic denitrification was measured in the oxygenated, organic-poor, sandy sediments of the Georgia continental shelf. Rates of denitrification were found to be low in the Georgia sands, as expected, but these rates would be significant globally if extrapolated over all sandy, continental shelf sediments. Heterotrophic denitrification was found to be the sole pathway of fixed N removal in these environments. The O_2 effect, an instrument-specific phenomenon in which O_2 and N_2 interact within the ion source of the mass spectrometer to form NO^+, was examined. This effect can adversely affect N_2 measurements and subsequent denitrification estimates. An optimal flow rate was determined that minimized the O_2 effect yet maintained analytically reasonable sampling times. Lastly, N_2 fixation and denitrification were simultaneously measured in Georgia salt marsh sediments during the summer and winter. The mechanism of denitrification varies with the season and is consistent with previous findings of seasonal variations of anaerobic respiration rates. Rates of both denitrification and nitrogen fixation are low and almost equal. These findings suggest that denitrification and N_2 fixation balance one another

internally, preventing large fluxes of N from entering or leaving salt marsh ecosystems. The results of these studies show that direct measurements of N cycling processes are vital to understanding the nuances of local N cycling.

CHAPTER 1

INTRODUCTION

1.1 Marine Nitrogen

All living organisms require the nutrient nitrogen (N) for the production of amino acids, the primary constituents of compounds such as proteins and nucleotides, which are vital to cellular function. The availability of biologically useable forms of N in marine environments plays a major role in controlling global productivity. In turn, marine productivity controls the amount of organic carbon ultimately buried in sediments, which on geologic time scales modulates the oxygen (O_2) and carbon dioxide (CO_2) contents of the atmosphere (Holland, 1984). Over 95% of total marine N is not available to most organisms (Rosswall, 1983), leaving the bioavailable, or fixed, N in short supply. The supply and removal of fixed marine N is controlled by various biotically-mediated reduction and oxidation reactions.

Marine N can be separated into two categories: fixed and non-bioavailable N. Fixed N is nitrogen that most organisms can assimilate into new biomass and in the ocean is composed of the inorganic N species ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), and certain organic N species. These species are restricted to N that is not bonded to another N atom (Chameides, 1997). The vast majority of the fixed N in the ocean is in the form of NO_3^-. Non-bioavailable N is composed of dissolved dinitrogen (N_2) and

nitrous oxide (N_2O) gases, which cannot be assimilated by most marine biota, except for a small group of organisms that can convert N_2 into fixed forms.

1.2 The Marine N Cycle

The various forms of N in the ocean are involved in biological and chemical reactions that comprise the marine N cycle. Three key biotically mediated reactions, nitrification, denitrification, and N_2 fixation, are the main controls of fixed N abundance in the global ocean (Figure 1.1).

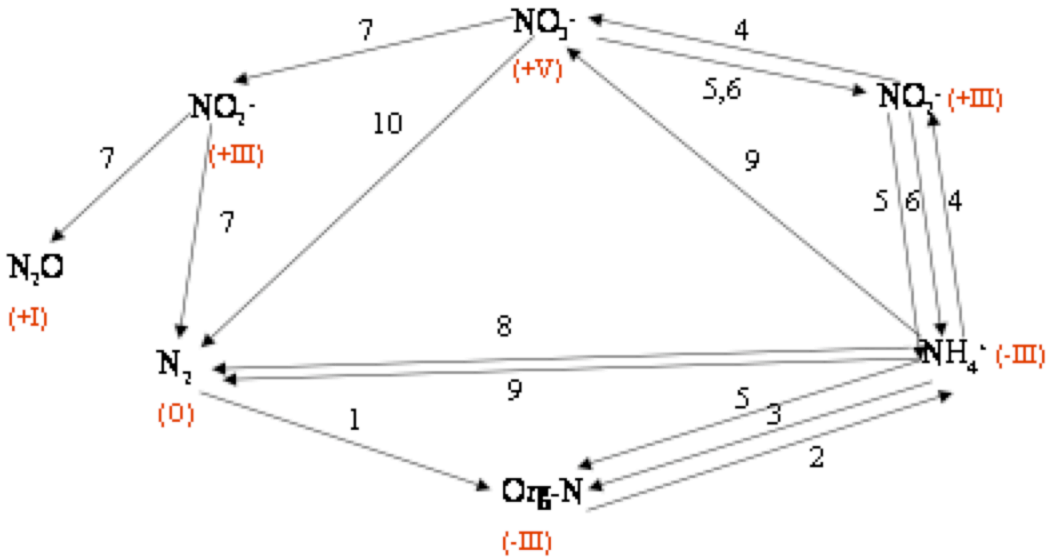

Figure 1.1: Schematic view of the biotic part of the biogeochemical N cycle. Adapted from Soderlund and Rosswall, 1982. The oxidation state of N is shown in red. The numbers listed on the figure represent the following processes: 1. Nitrogen fixation. 2. Mineralization. 3. Immobilization (ammonia assimilation). 4. Nitrification. 5. Immobilization (nitrate and nitrite assimilation). 6. Non-assimilatory nitrate reduction. 7. Denitrification. 8. Anammox. 9. Ammonium oxidation with MnO_2. 10. Nitrate reduction with Mn^{2+}.

1.2.1 Canonical heterotrophic denitrification

The main removal mechanism of marine fixed N is denitrification. Denitrification describes the conversion process of inorganic N species, primarily NO_3^-, into N_2 (pathway 7, Figure 1; heterotrophic denitrification reaction shown in equation (1.1)):

$$(CH_2O)_{106}(NH_3)_{16}(H_3PO_4) + 94.4HNO_3 \rightarrow 106CO_2 + 55.2N_2 + H_3PO_4 + 177.2H_2O \quad (1.1)$$

During this microbially mediated process, NO_3^- is used as an electron acceptor during organic matter oxidation in areas of little or no O_2 such as anoxic basins and sediments. This classical view of denitrification is referred to as canonical heterotrophic denitrification. Heterotrophic organisms acquire energy through the catabolic breakdown of organic matter. Denitrifying bacteria are facultative anaerobes, meaning that they oxidize organic matter using O_2 because the highest amount of free energy is yielded when O_2 is used as an oxidant (Froelich, 1979). Under anaerobic conditions, facultative anaerobic bacteria can utilize other electron acceptors, such as NO_3^- and sulfate (SO_4^{2-}), based on thermodynamics and availability. Denitrifying bacteria also produce small amounts of N_2O during denitrification (Gardner, 1987; Laursen, 2002). However, N_2O will not be considered in this study.

1.2.2 Alternative fixed N removal processes

Denitrification is not the only mechanism through which fixed N is removed. NH_4^+ can be anaerobically oxidized to N_2 in the absence of organic matter (autotrophically) through

the "anammox" (anaerobic ammonium oxidation) reaction (Mulder, 1995; Jetten, 1999; Dalsgaard, 2003; Kuypers, 2003):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad (1.2)$$

This process was known to occur in wastewater treatment systems and bioreactors (Strous, 1999; Kuenen, 2001) and has also been discovered in nature (Dalsgaard, 2003; Kuypers, 2003). A recent examination of anammox revealed that it could possibly account for between 30% and 50% of water column N_2 production (Devol, 2003). However, its significance in marine sediments continues to be investigated, globally, by many groups of scientists.

Marine fixed N can be further removed through the anaerobic oxidation of ammonium by manganese dioxide (MnO_2) via two pathways:

$$4MnO_2 + NH_4^+ + 6H^+ \rightarrow 4Mn^{2+} + NO_3^- + 5H_2O \qquad (1.3a)$$

$$3MnO_2 + 2NH_4^+ + 4H^+ \rightarrow 3Mn^{2+} + N_2 + 6H_2O \qquad (1.3b)$$

Reaction (1.3a) indirectly removes fixed N by converting NH_4^+ to NO_3^-, which can be subsequently denitrified to form N_2. The Mn cycle interacts with the N cycle through yet another reaction involving the reduction of nitrate by Mn^{2+}:

$$5Mn^{2+} + 2NO_3^- + 4H_2O \rightarrow 5MnO_{2(solid)} + N_2 + 8H^+ \qquad (1.3c)$$

The coupling of the manganese cycle to the nitrogen cycle has been cited as an alternative means of fixed N removal and N_2 production (Sorensen, 1987; Aller, 1990; Murray, 1995; Luther, 1997; Hulth, 1999; Newton, 2006).

1.2.3 Nitrification

Another process within the N cycle is nitrification, the oxidation of ammonium, NH_4^+, which results in the formation of NO_2^- and/or NO_3^-. This process is carried out by two different classes of organisms: *Nitrosomonas* bacteria, which catalyze the oxidization of NH_4^+ to form NO_2^-, and *Nitrobacter*, which catalyze the oxidation of nitrite to nitrate (reactions shown in equations (1.4a) and (1.4b)):

$$NH_4^+ + \tfrac{3}{2}O_2 \rightarrow NO_2^- + H_2O + 2H^+ \qquad (1.4a)$$

$$NO_2^- + \tfrac{1}{2}O_2 \rightarrow NO_3^- \qquad (1.4b)$$

Nitrification is thought to be coupled to denitrification in marine sediments (Jenkins, 1984). Evidence of this coupling comes through data suggesting that measured NO_3^- concentrations in some regions are too low to support observed rates of denitrification (Codispoti, 1985; Laursen, 2002). The NO_3^- required for denitrification in these regions may be supplied through nitrification. The mechanism of coupled nitrification-denitrification can be represented by the following equations:

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+ \qquad (1.5a)$$

$$5CH_2O_{(organicmatter)} + 4NO_3^- + 4H^+ \rightarrow 2N_2 + 5CO_2 + 7H_2O \qquad (1.5b)$$

Nitrification, in this case, is an aerobic process (as opposed to anaerobic ammonium oxidation by MnO_2, reaction (1.3a)). Hence, O_2 can be consumed during coupled nitrification-denitrification reactions. Although nitrification and denitrification reactions are coupled in some sedimentary environments, the exact locations within the sediment where each process takes place are separated based on oxygen availability. Nitrification can occur as far down in the sediment as the O_2 penetrates and denitrification usually occurs below the oxygen penetration depth in the suboxic and/or

anoxic layer(s). Denitrification rates have been found to be higher in sediments overlain by well-oxygenated bottom waters than in oxygen-depleted zones due to increased availability of NO_3^- produced during nitrification (Hartnett, 2003). Based on the higher overall rates and the spatial distribution of continental margins containing well-oxygenated bottom water, denitrification may be more significant in these sediments than in oxygen-depleted zones.

1.2.4 Nitrogen fixation

Nitrogen fixation describes the process in which nitrogen gas is converted to fixed N species primarily through biologically mediated reactions, schematically represented in equation (1.6):

$$N_2 + 5H_2O \rightarrow 2NH_4^+ + 2OH^- + \tfrac{3}{2}O_2 \qquad (1.6)$$

Biological N_2 fixation is restricted to a limited group of organisms. The microorganisms that perform this process are called diazotrophs. *Trichodesmia* are thought to be the most abundant diazotrophic bacteria in the ocean (Falcon, 2002). However, diazotrophs consist of different types of bacteria, some of which are photosynthetic, such as *Trichodesmia*, while others are heterotrophic. Heterotrophic diazotrophs, specifically, nitrogen-fixing sulfate-reducing bacteria, have been identified and studied in the marine environment for over 50 years (Sisler, 1951; Jones, 1974; Lovell, 2001). Bacteria belonging to the genus *Desulfovibrio* were isolated and incubated to provide evidence for anaerobic N_2 fixation (Sisler, 1951; Steppe, 2002). Experimental data presented by Sisler and ZoBell (1951) showed that these bacteria are able to fix nitrogen through the electron transfer between H_2 (electron donor) and the sulfur (electron acceptor) in H_2SO_4. Other

sulfate-reducing bacteria such as *Azotobacter* and *Clostridium* have also shown potential for fixing nitrogen (Herbert, 1975; Nielsen, 2001; Ravikumar, 2004). *Desulfovibrio* has been isolated from deep-sea sediments (Sisler, 1951) and was found to be responsible for *in-situ* N_2 fixation in estuarine sediments (Jones, 1974; Herbert, 1975; Kurtz, 1998; Steppe, 2002).

Unicellular cyanobacteria have also been found to support substantial rates of N_2 fixation (Dore, 2002; Montoya, 2004; Needoba, 2007; Staal, 2007). *Trichodesmia* are photosynthetic diazotrophic bacteria, therefore N_2 fixation rates associated with the organism are found to be higher during the day (Staal, 2007). In some areas of the eastern Atlantic Ocean, diazotrophic activity was found to be highest at night and, therefore, suspected to be the result of N_2 fixation by organisms other than *Trichodesmia*, specifically unicellular cyanobacteria such as *Crocosphaera* (Staal, 2007).

Montoya et al. (2004) determined that unicellular diazotrophic bacteria are producing new nitrogen in the oligotrophic Pacific Ocean at rates that are comparable to inputs from *Trichodesmia* and *Richelia*, a symbiotic N_2-fixing bacteria (Carpenter, 1999). One potential reason that unicellular cyanobacteria are responsible for such high rates of N_2 fixation is their distribution throughout the water column, unlike *Trichodesmia* that reside mostly on the surface of the ocean because of their buoyancy.

Inputs of fixed N, primarily by means of nitrogen fixation, should be balanced with fixed N removal rates, mainly denitrification, in order to maintain steady-state concentrations of fixed N in the global N reservoirs. However, current estimates of denitrification exceed those of N_2 fixation, suggesting that biologically available N is being lost to the atmosphere as N_2 (Christensen, 1987; Nixon, 1996; Seitzinger, 1996;

Pilson, 1998; Laursen, 2002). Three plausible explanations for the unbalanced, marine fixed N budget (Figure 1.2) are: 1.) N_2 fixation has been underestimated; 2.) Denitrification has been overestimated; 3.) Both processes have been miscalculated.

1.2.5 Rates – N_2 fixation and denitrification

Biological N_2 fixation is the primary means by which fixed N is supplied to the ocean. However, N_2 fixation is limited to a small group of organisms, one of the most abundant being *Trichodesmium*. *Trichodesmia* are cyanobacteria that inhabit surface waters of oligotrophic, tropical, and sub-tropical oceans (Carpenter, 1983). N_2 fixation can be estimated by the abundance of *Trichodesmium*

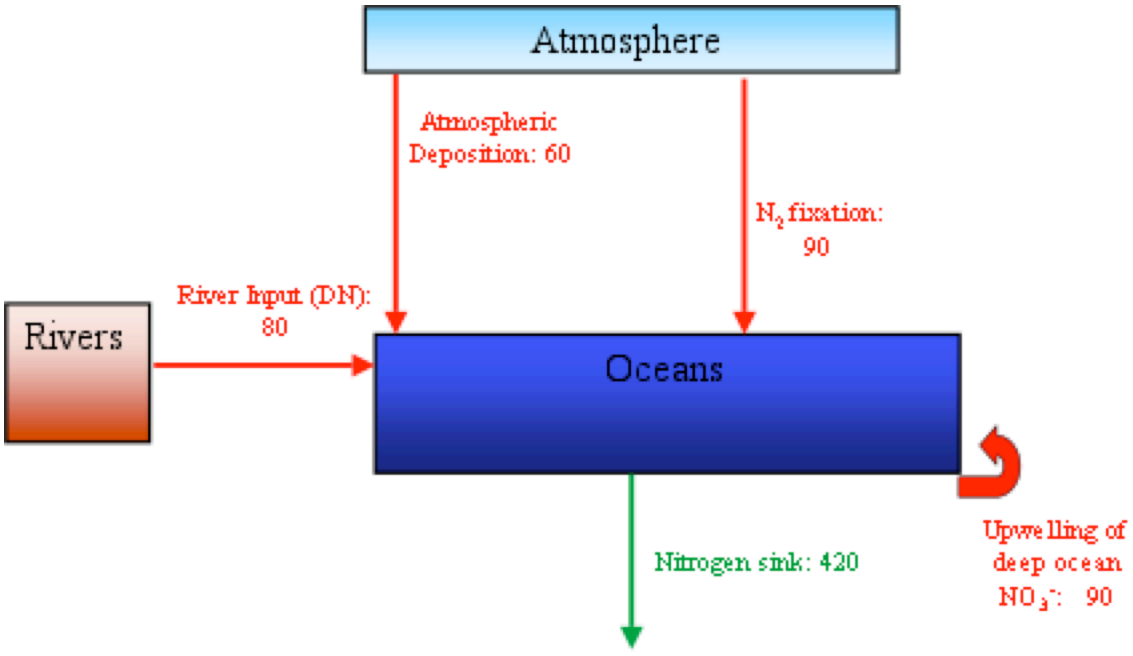

Figure 1.2: Fluxes of marine fixed N. All values taken from Kolber, 2006. All fluxes have units of Tg N/yr. Tg = 10^{12}g. The term, nitrogen sink, is presumably referring to denitrification and sedimentation. The paper from which these values were taken focuses on the increased fixation term, arising from the discovery of a new technique to identify *Trichodesmia* in 3-D, which resulted in increased fixation rates. There is still a missing source of fixed N in the ocean, which may account for the 100 Tg N/yr deficit.

found in a sample and the rate at which colonies of this organism grow (Carpenter, 1977; McCarthy, 1979; Lipschultz, 1996; Karl, 1997). Global fixation rates have been obtained by estimating colonial abundances, based on collected samples, and extrapolating fixation rates over the global oceans (Lipschultz, 1996). Recently, a new imaging technique was developed to identify *Trichodesmia* abundances in 3-D (Davis, 2006). The use of this technique in the North Atlantic Ocean resulted in increased global N_2 fixation rates, which may account for part of the estimated deficit of fixed N sources (Kolber, 2006).

N_2 fixation has also been estimated using a variety of techniques. Some studies have investigated the increase in dissolved organic nitrogen (DON) in surface waters and have attributed the increase to nitrogen fixation (Hansell, 1997; Vidal, 1999; Abell, 2000; Hansell, 2000). Other studies have interpreted low ^{15}N abundances in organic matter found in surface waters as an indication of biological N_2 fixation because their isotopic signatures were similar to those found in N in the atmosphere (Macko, 1984; Carpenter, 1997; Karl, 1997; Brandes, 1998; Capone, 1998; Carpenter, 1999). It is possible that N_2 fixation has been underestimated due to insufficient information on *Trichodesmium* (Capone, 1997) and other N_2 fixers (Capone, 2001) as well as other techniques in evaluating N_2 fixation.

The proposed imbalance in global N budgets (Figure 1.2) also suggests that denitrification rates may have been overestimated. Seitzinger and Giblin (1996) obtained denitrification rate estimates by extrapolating estimates made in several environments to North Atlantic continental shelf sediments. These denitrification rate estimates were based on relationships between primary production (PP), sediment oxygen demand

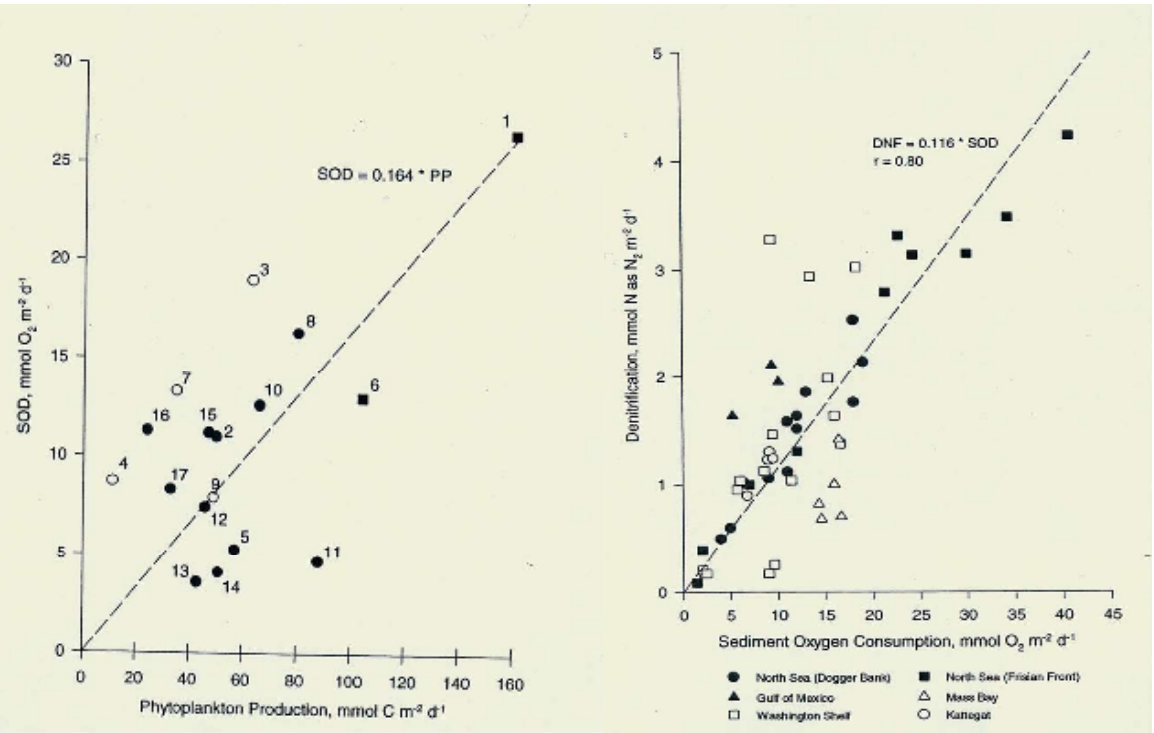

Figure 1.3: Reprinted from Seitzinger and Giblin, 1996. Relationships between phytoplankton production (PP) with sediment oxygen demand (SOD) and SOD with denitrification. These relationships were used to estimate denitrification rates in a variety of continental shelf regions. The relationship between SOD and PP is not well-correlated, which may have resulted in inaccurate estimates of global denitrification rates.

(SOD) and denitrification rates (Figure 1.3). However, their established relationship between SOD and PP was not well correlated. Hence, the denitrification rates that were obtained from this relationship are not well-constrained.

Stoichiometric and model-based estimates are inferior to direct measurements because processes such as NO_3^- diffusion, coupled nitrification-denitrification reactions, and macrobenthic irrigation are not considered (Devol, 1991). There is an experimental technique used to measure denitrification that employs acetylene, which blocks the microbial reduction of N_2O to N_2, the final end product of denitrification, and measures denitrification activity by the amount of N_2O produced (Blackburn, 1986; Koike, 1988; Christensen, 1989). This method does not consider that N_2O has been invoked as a minor

intermediate and/or product of nitrification (Yoshida, 1970; Ritchie, 1972; Blackmer, 1980; Goreau, 1980). Therefore, all of the relevant N species in a particular environment should be considered when studying any aspect of the nitrogen cycle.

Some denitrification rate estimates have been based on direct measurements. Pore-water NO_3^- profiles have been used to determine denitrification based on the rate of disappearance of NO_3^- (Bender, 1977; Aller, 1985; Christensen, 1987). Because NO_3^- can be assimilated by marine organisms as well as denitrified, the disappearance of NO_3^- is not a sole indicator of denitrification. However, it can be used along with the N_2 flux method for mass balance purposes (Devol, 1993). The N_2 flux method measures the flux of N_2 gas between the overlying water and the sediments (Devol, 1993; Devol, 1997). The difficulty in this method is the actual measurement of N_2 gas. The development of membrane inlet mass spectrometry has increased the accuracy of this method (Kana, 1994). These and other direct denitrification measurements are, however, limited to a few sampling locations. It is imperative that direct denitrification rate measurements are made in as many locations as possible in order to account for spatial variability and to determine the role of processes such as coupled nitrification-denitrification and anammox.

1.3 Membrane Inlet Mass Spectrometry (MIMS)

Information on denitrification rates in all marine environments has been hampered by the complexity of measuring dissolved N_2 in natural waters. Previous investigations of denitrification rates have utilized a variety of techniques including stoichiometric calculations, gas chromatography, acetylene block, and *in situ* measurements using

benthic chambers (Seitzinger, 1980; Seitzinger, 1983; Devol, 1991; Blackburn, 1993; Devol, 1993; Christensen, 1996; Marinelli, 1998; van Luijn, 1999; Laursen, 2002). A relatively new technique, membrane inlet mass spectrometry (MIMS), was developed that measures dissolved gases in natural samples (Kana, 1994). The instrument can detect masses 28, 29, and 30, which can be attributed to various combinations of the stable isotopes of N (^{14}N, ^{15}N) in N_2. Additionally, other masses can be detected using MIMS such as mass 40, which can be attributed to argon (Ar) gas and mass 32, which can be attributed to O_2. Mass 28 can also be attributed to carbon monoxide (CO) gas, a fragment of CO_2 gas. A liquid N_2 trap is used to remove water vapor and CO_2, preventing interference of CO with $^{28}N_2$. MIMS is a significant advance in the measurement of dissolved gases because there is no degassing step involved, as is necessary in the gas chromatography technique, a smaller sample size can be used (8-10 mL), measurements can be made in a more timely fashion (20 – 30 samples/hr), and it is extremely precise (<0.05% error for N_2/Ar analyses) (Kana, 1994).

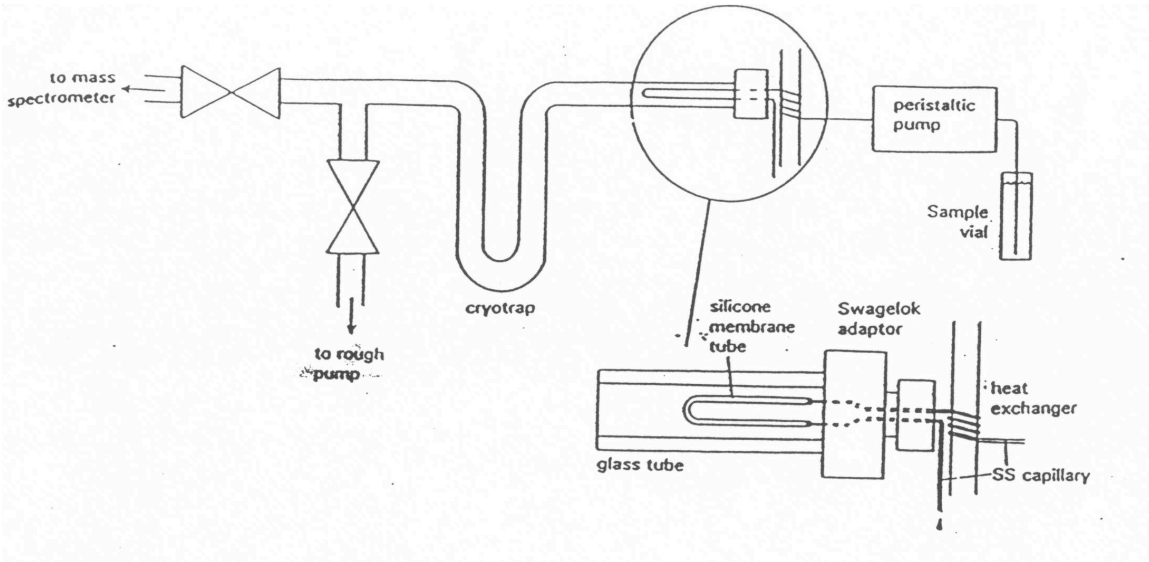

Figure 1.4: Reproduced from Kana et al., 1994. Schematic representation of the MIMS sample pumping and vacuum interface to the quadrupole mass spectrometer.

MIMS consists of a water pumping system that allows water to flow from the sample to the membrane inlet via the use of a peristaltic pump and stainless steel (SS) capillary tubing (Figure 1.4). The sample water flows through a heat exchanger, used to stabilize the temperature of the thermostated water, and into the silicone membrane tubing, which serves as a semipermeable membrane interface that allows diffusion of dissolved gases. The gases exit the SS capillary tubing and go through the vacuum inlet system, which consists of the liquid N_2 cryotrap discussed above. The gas sample then continues to the quadrupole mass spectrometer where masses 28, 29, 30, 32, and 40 are detected (Figure 1.4) (Kana, 1994). The output signal obtained from MIMS is given in units of amplitudes (A).

MIMS experiences some drift in the output signal during sample analysis. Several steps are required to correct for drift in this instrument. Two water baths are set at different temperatures, 21°C and 30°C, and a flask of bottom water obtained from the study site is placed in each water bath and allowed to equilibrate with the atmosphere. These seawater solutions are used as standards and analyzed for dissolved gases periodically during the analyses of the natural samples. Solubility equations are used to calculate the expected concentrations of each gas, based on the water temperature and salinity.

1.4 Global Marine N Budget

Global marine fixed N budgets have been attempted and proposed by many scientists over the past few decades. It is still unclear whether or not the N budget is balanced. Many studies, and scientists alike, have indicated that the N budget is out of balance, with

rates of losses exceeding rates of input. As measurement techniques improve and more direct measurements are made, rates of denitrification seem to increase. There have also been key discoveries of reactions that consume fixed N, resulting in N_2 production, namely anammox and Mn-catalyzed nitrification and denitrification. The combination of these advancements in our understanding of fixed N removal mechanisms have led many scientists to believe that the global marine fixed N budget is out of balance.

A recent study (Brandes, 2007) has proposed a N budget that may be balanced. The authors were able to propose a possible balanced budget by surmising that the biological N_2 fixation term must be on the order of the upper limits of previously proposed rates. Biological N_2 fixation has primarily been estimated using the cyanobacterial species *Trichodesmium* as a tracer. As more information is gathered on the spatial distribution and activity levels of *Trichodesmium* as well as other significant N_2 fixers, especially heterotrophic N_2-fixing bacteria, rates of biological N_2 fixation may indeed be higher than previously thought.

There is substantial evidence in support of the natural processes regulating the marine N cycle occurring at greater rates than was originally proposed (Galloway, 2004). In conjunction with an increased level of understanding of the processes that are occurring are the changes in the rates of these processes due to anthropogenic activity. Galloway et al. (2004) have gathered a vast amount of previously published data on N cycling rates and have re-analyzed data to propose new rates in order to create budgets for 1860, i.e. pre-industrial conditions, early 1990s, i.e. current conditions, and 2050. The most significant changes in the N budget are the result of the impact of anthropogenic activity. N_2 fixation from anthropogenic activity results in a doubling of

rates of fixation from 1860 to 2050. Also, atmospheric emissions and deposition rates as well as riverine fluxes dramatically increase. The results of their study suggest that the impact of human activity may result in an overabundance of fixed N in less than 50 years from now.

1.5 Organization and Content of the Dissertation

Based on the evidence from the literature, it seems that more information and direct measurements are needed for both the input and loss terms of the marine N budget. As Brandes et al. (2007) suggest, the integrated rates of these processes are necessary to obtaining a more accurate global N budget, more than just the discoveries of new reactions in new places.

As much as 70% of continental shelf sediments are composed of coarse sands (Emery, 1968). It is well understood that the most globally significant site of heterotrophic denitrification, the primary loss term, is the continental shelf (Codispoti, 1985; Christensen, 1987; Pilson, 1998). Direct measurements of denitrification in coarse-grained continental shelf sediments are especially vital for achieving accurate rates on a global scale. Presented in chapter 2 of this dissertation are results of direct measurements of denitrification in coarse-grained, sandy sediments within the continental shelf of Georgia, USA.

Membrane inlet mass spectrometry (MIMS) techniques are used throughout the work that is presented in this dissertation. This technique has been used for denitrification measurements for ca. 15 years. There has been some debate in the literature on what effect, if any, dissolved O_2 has on N_2 measurements using MIMS

(Eyre, 2002; Eyre, 2004; Kana, 2004). The results presented in chapter 3 offer some insight into the cause for differences in the response of the MIMS to dissolved O_2 concentrations.

Heterotrophic N_2 fixation is another facet of the N cycle that has been overlooked. This process could represent a significant source of fixed N to the marine system. More information on distribution and rates of heterotrophic N_2 fixation could potentially improve current estimates of global fixation rates. Heterotrophic N_2 fixation and denitrification was observed in a Georgia salt marsh and the results of a seasonal study using salt marsh sediments are presented in chapter 4.

A summary of the results presented in chapters 2-5 is included in the conclusions, along with future research directions.

1.6 References

Abell, J., Emerson, S., and Renaud, P. (2000) "Distibutions of TOP, TON and TOC in the North Pacific subtropical gyre: Implications for nutrient supply in the surface ocean and remineralization in the upper thermocline." *Journal of Marine Reseach*. 58, 203-222.

Aller, R. C. (1990) "Bioturbation and manganese cycling in hemipelagic sediments." *Philosophical Transactions of the Royal Society of London Series B - Biological Sciences*. 331, 51-68.

Aller, R. C., Mackin, J. E., Ullman, W. J., Wang, C.-H., Tsai, S.-M., Jin, J.-C., Sui, Y.-N., Hong, J. Z. (1985) "Early chemical diagenesis, sediment-water exchange, and storage of reactive organic matter near the mouth of the Changjiang, East China Sea." *Continental Shelf Research*. 4(1/2), 227-251.

Bender, M. L., Fanning, K. A., Froelich, P. N., Heath, G. R., and Maynard, V. (1977) "Interstitial nitrate profiles and oxidation of sedimentary organic matter in the eastern equatorial Atlantic." *Science*. 198, 605-609.

Blackburn, N. D., Blackburn, T.H. (1993) "A reaction diffusion model of C-N-S-O species in a stratified sediment." *FEMS Microbiology Ecology*. 102, 207-215.

Blackburn, T. H. (1986) "Nitrogen cycle in marine sediments." *Opheila*. 26, 65-76.

Blackmer, A. M., Bremner, J. M., and Schmidt, E. L. (1980) "Production of nitrous oxide by ammonia oxidizing chemoautotrophic microorganisms in soil." *Applied and Environmental Microbiology*. 40, 1060-1066.

Brandes, J., Devol, A., Yoshinari, T., Jayakumar, D., and Naqvi, S. (1998) "Isotopic composition if nitrate in the central Arabian Sea and eastern tropical North Pacific: a tracer for mixing and nitrogen cycles." *Limnology and Oceanography*. 43, 1680-1689.

Brandes, J. A., Devol, Allan H. and Deutsch, Curtis. (2007) "New Developments in the marine nitrogen cycle." *Chemical Reviews*. 107, 577-589.

Capone, D. G. (2001) "Marine nitrigen fixation: what's the fuss?" *Current Opinion in Microbiology*. 4, 341-348.

Capone, D. G., Subramaniam, A., Montoya, J., Voss, M., Humborg, C., Johansen, A., Siefert, R., and Carpenter, E. J. (1998) "An extensive bloom of the N_2-fixing cyanobacterium, *Trichodesmium erythraeum*, in the Central Arabian Sea." *Marine Ecology Progress Series*. 172, 281-292.

Capone, D. G., Zehr, J. P., Paerl, H. W., Bergman, B., and Carpenter, E. J. (1997) "*Trichodesmium*: a globally significant cyanobacterium." *Science*. 276, 1221-1229.

Carpenter, E. J. (1983) "Physiology and ecology of marine planktonic *Oscillatoria (Trichodesmium)*." *Marine Biology Letters*. 4, 69-85.

Carpenter, E. J., Harvey, H. G., Fry, B., Capone, D. G. (1997) "Biogeochemical tracers of the marine cyanobacterium *Trichodesmium*." *Deep-Sea Research*. 44, 27-38.

Carpenter, E. J., Montoya, J. P., Burns, J., Mulholland, M., Subramaniam, A., and Capone, D. G. (1999) "Extensive bloom of a N_2 fixing symbiotic association in the tropical Atlantic Ocean." *Marine Ecology Progress Series*. 188, 273-283.

Carpenter, E. J., Price, C.C. (1977) "Nitrogen fixation, distibution, and production of *Oscillatoria (Trichodesmium)* spp. in the western Sargasso and Caribbean Seas." *Limnology and Oceanography*. 22, 60-72.

Chameides, W. L., Perdue, E. M. (1997). *Biogeochemical Cycles: A Computer-Interactive Study of Earth System Science and Globgal Change*. Oxford University Press, Inc.

Christensen, J. P., Murray, J. M., Devol, A. H., and Codispoti, L. A. (1987) "Denitrification in continental shelf sediments has a major impact on the oceanic nitrogen budget." *Global Biogeochemical Cycles*. 1(2), 97-116.

Christensen, J. P., Townsend, D. W. and Montoya, J. P. (1996) "Water column nutrients and sedimentary denitrification in the Gulf of Maine." *Continental Shelf Research*. 16(4), 489-515.

Christensen, P. B., Nielsen, L. P., Revsbech, N. P., and Sorensen, J. (1989) "Microzonation of Denitrification Activity in Stream Sediments as Studied with a Combined Oxygen and Nitrous Oxide Microsensor." *Applied and Environmental Microbiology*. 55(5), 1234-1241.

Codispoti, L. A., Christensen, J. P. (1985) "Nitrification, denitrification, and nitrous oxide cycling in the eastern tropical South Pacific Ocean." *Marine Chemistry*. 16, 277-300.

Dalsgaard, T., Canfield, D. E., Petersen, J., Thamdrup, B., Acuna-Gonzalez, J. (2003) "N_2 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica." *Nature*. 422, 606-608.

Davis, C. S. a. M., D. J. (2006) "Transatlantic abundance of the N-2 fixing cyanobaterium *Trichodesmium*." *Science*. 312(5779), 1517-1520.

Devol, A. H. (1991) "Direct measurement of nitrogen gas fluxes from continental shelf sediments." *Nature*. 349(6307), 319-321.

Devol, A. H. (2003) "Solution to a marine mystery." *Nature*. 422, 575-576.

Devol, A. H., Christensen, J. P. (1993) "Benthic fluxes and nitrogen cycling in sediments of the continental margin of the eastern North Pacific." *Journal of Marine Research*. 51, 345-372.

Devol, A. H., Codispoti, L. A., and Christensen, J. P. (1997) "Summer and winter denitrification rates in western Arctic shelf sediments." *Continental Shelf Research*. 17, 1029-1050.

Dore, J. E., Brum, J. R., Tupas, L., and Karl, D. M. (2002) "Seasonal and interannual variability in sources of nitrogen supporting export in the oligotrophic subtropical North Pacific Ocean." *Limnology and Oceanography*. 47, 1595-1607.

Emery, K. O. (1968) "Relict sediments on continental shelves of the world." *American Association of Petrology and Geology*. 52, 445-464.

Eyre, B. D., Rysgaard, S., Dalsgaard, T., Christensen, P. B. (2002) "Comparison of Isotope Pairing and N2:Ar Methods for Measuring Sediment Denitrification-Assumptions, Modifications, and Implications." *Estuarines*. 25, 1077-1087.

Eyre, B. D., Rysgaard, S., Dalsgaard, T., Christensen, P. B. (2004) "Reply to Comment on our paper "Comparison of isotope pairing and N_2:Ar methods for measuring sediment denitrification"." *Estuaries*. 27, 177-178.

Falcon, L. I., Cipriano, F., Christoserdov, A. Y., Carpenter, E. J. (2002) "Diversity of diazotrophic unicellular cyanobacteria in the tropical North Atlantic Ocean." *Applied and Environmental Microbiology*. 68(11), 5760 - 5764.

Froelich, P. N., Klinkhammer, G.P., Bender, M.L., Luedtke, N.A., Heath, G.R., Cullen, D., Dauphin, P., Hammond, D., Hartman, B., and Maynard, V. (1979) "Early oxidation of organic matter in pelagic sediments of the eastern equatorial Atlantic - Suboxic diagenesis." *Geochemica et Cosmochimica Acta*. 43(7), 1075-1090.

Galloway, J. N., Dentener, F.J., Capone, D.G., Boyer, E.W., Howarth, R.W., Seitzinger, S.P., Asner, G.P., Cleveland, C.C., Green, P.A., Holland, E.A., Karl, D.M., Michaels, A.F., Porter, J.H., Townsend, A.R. and Vorosmarty, C.J. (2004) "Nitrogen cycles: past, present, and future." *Biogeochemistry*. 70(2), 153-226.

Gardner, W. S., Nalepa, T. F., and Malczyk, J. M. (1987) "Nitrogen mineralization and denitrification in Lake Michigan sediments." *Limnology and Oceanography*. 32(6), 1226-1238.

Goreau, T. J., Kaplan, W.A., Wofsy, S.C., McElroy, M.B., Valois, F.W. and Watson, S.W. (1980) "Production of NO_2^- and N_2O by nitrifying bacteria at reduced concentrations of oxygen." *Applied and Environmental Microbiology*. 40, 526-532.

Hansell, D., Feely, R. (2000) "Atmospheric intertropical convergence impacts surface ocean carbon and nitrogen biogeochemistry in the western tropical Pacific." *Geophysical Research Letters*. 27, 1013-1016.

Hansell, D., Waterhouse, T. Y. (1997) "Controls on the distribution of organic carbon and nitrogen in the eastern Pacific ocean." *Deep-Sea Research Part I*. 44, 843-857.

Hartnett, H. E., Devol, A.H. (2003) "Role of a strong oxygen-deficient zone in the preservation and degradation of organic matter: A carbon budget for the continental margins of northwest Mexico and Washington State." *Geochimica et Cosmochimica Acta*. 67(2), 247-264.

Herbert, R. A. (1975) "Heterotrophic nitrogen fixation in shallow estuarine sediments." *Journal of Experimental Marine Biology and Ecology*. 18, 215-225.

Holland, H. D. (1984). *The Chemical Evolution of the Atmosphere and Oceans*. Princenton University Press

Hulth, S., Aller, R. C., and Gilbert, F. (1999) "Coupled anoxic nitrification/manganese reduction in marine sediments." *Geochimica et Cosmochimica Acta*. 63(1), 49-66.

Jenkins, M. C., Kemp, W. M. (1984) "The coupling of nitrification and denitrification in two estuarine sediments." *Limnology and Oceanography*. 29, 609-619.

Jetten, M. S. M., Strous, M., van de Pas-Schoonen, K. T., Schalk, J., van Dongen, G. J. M., van de Graaf, A. A., Logemann, S., Muyzer, G., van Loosdrecht, M. C. M., Kuenen, J. G. (1999) "The anaerobic oxidation of ammonium." *FEMS Microbiology Reviews*. 22, 421-437.

Jones, K. (1974) "Nitrogen fixation in a salt marsh." *The Journal of Ecology*. 622, 553-565.

Kana, T. M., Darkangelo, C., Hunt, M. D., Oldham, J. B., Bennett, G. E. and Cornwell, J. C. (1994) "Membrane inlet mass spectrometry for rapid high-precision determination of N_2, O_2, and Ar in environmental water samples." *Analytical Chemistry*. 66(23), 4166-4170.

Kana, T. M. a. W., D. L. (2004) "Comment on "Comparison of isotope pairing and N_2:Ar methods for measuring sediment denitrification" By B. D. Eyre, S. Rysgaard, T. Dalsgaard, and P. Bondo Christensen. *Estuaries* 25: 1077-1087." *Estuaries*. 27, 173-176.

Karl, D., Letelier, R., Tupas, L., Dore, J., Christian, J., and Hebel, D. (1997) "The role of nitrogen fixation in biogeochemical cycling in the subtropical North Pacific Ocean." *Nature*. 388, 533-538.

Koike, I., Sorensen, J. (1988) Nitrate reduction and dentrification in marine sediments. *Nitrogen cycling in coastal marine environments*. (T. H. Blackburn, Sorensen, J.), Wiley.

Kolber, Z. S. (2006) "Ecology - Getting a better picture of the ocean's nitrogen budget." *Science*. 312(5779), 1479-1480.

Kuenen, J. G., Jetten, M. S. M. (2001) "Extraordinary anaerobic ammonium-oxidizing bacteria." *American Society for Microbiology News*. 67, 456-463.

Kurtz, J. C., Devereux, R., Barkay, T. and Jonas, R. B. (1998) "Evaluation of sediment slurry microcosms for modeling microbial communities in estuarine sediments." *Environmental Toxicology ad Chemistry*. 17(7), 1274-1281.

Kuypers, M. M. M., Sliekers, A. O., Lavik, G., Schmid, M., Jorgensen, B. B., Kuenen, J. G., Damste, J. S. S., Strous, M., Jetten, M. S. M. (2003) "Anaerobic ammonium oxidation by anammox bacteria in the Black Sea." *Nature*. 422, 608-611.

Laursen, A. E., Seitzinger, S. P. (2002) "The role of denitrification in nitrogen removal and carbon mineralization in Mid-Atlantic Bight sediments." *Continental Shelf Research*. 22, 1397-1416.

Lipschultz, F., Owens, N. J. P. (1996) "An assessment of nitrogen fixation as a source of nitrogen to the North Atlantic Ocean." *Biogeochemistry*. 35(1), 261-274.

Lovell, C. R., Friez, Michael J., Longshore, John W. and Bagwell, Christopher E. (2001) "Recovery and phylogenetic analysis of *nifH* Sequences from diazotrophic bacteria associated with dead aboveground biomass of *Spartina alterniflora*." *Applied and Environmental Microbiology*. 67(11), 5308-5314.

Luther, G. W., Sundby, B., Lewis, B. L., Brendel, P. J., and Silverberg, N. (1997) "Interactions of manganese with the nitrogen cycle: Alternative pathways to dinitrogen." *Geochimica et Cosmochimica Acta*. 61(19), 4043-4052.

Macko, S. A., Enzeroth, L., Parker, P. L. (1984) "Regional differences in nitrogen and carbon isotopes on the continental shelf of the Gulf of Mexico." *Naturwissenschaften*. 71, 374-375.

Marinelli, R. L., Jahnke, R.A., Craven, D.B., Nelson, J.R., and Eckman, J.E. (1998) "Sediment nutrient dynamics on the South Atlantic Bight continental shelf." *Limnology and Oceanography*. 43(6), 1305-1320.

McCarthy, J. J., Carpenter, E. J. (1979) "*Oscillatoria (Trichodesmium) thiebautii (Cyanophyta)* in the central north Atlantic Ocean." *Journal of Phycology*. 15, 75-82.

Montoya, J. P., Holl, C.M., Zehr, .P., Hansen, A., Villareal, T.A. and Capone, D.G. (2004) "High rates of N-2 fixation by unicellular diazotrophs in the oligotrophic Pacific Ocean." *Nature*. 430(7003), 1027-1031.

Mulder, A., van de Graaf, A. A., Robertson, L. A., and Kuenen, J. G. (1995) "Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor." *FEMS Microbiology Ecology*. 16, 177-184.

Murray, J. W., Codispoti, L. A., and Friederich, G. E. (1995) The suboxic zone in the Black Sea. *Aquatic Chemistry: Interfacial and INterspecies Processes*. (C. P. Huang, O'Melia, C. R., and Morgan, J. J.): 157-176, American Chemical Society.

Needoba, J. A., Foster, R. A., Sakamoto, C., Zehr, J. P., Johnson, K. S. (2007) "Nitrogen fixation by unicellular diazotrophic cyanobacteria in the temperate oligotrophic North Pacific Ocean." *Limnology and Oceanography*. 52(4), 1317-1327.

Newton, J. D. (2006). Evidence for manganese-catalyzed nitrogen cycling in salt marsh sediments. School of Earth and Atmospheric Sciences. Atlanta, GA, Georgia Institute of Technology. Master of Science: 80.

Nielsen, L. B., Finster, K., Welsh, David T., Donelly, A., Herbert, Rodney A., de Wit, Rutger and Lomstein, Bente Aa. (2001) "Sulphate reduction and nitrogen fixation rates associated with roots, rhozomes and sediments from *Zostera noltii* and *Spartina maritima* meadows." *Environmental Microbiology*. 3(1), 63-71.

Nixon, S. W., Ammerman, J. W., Atkinson, P., Berounsky, V. M., Billen, G., Boicourt, W. C., Boyton, W. R., Church, T. M., Ditoro, D. M., Elmgren, R., Garber, J. H., Giblin, A. E., Jahnke, R. A., Owens, N. J. P., Pilson, M. E. Q., and Seitzinger, S. P. (1996) "The fate of nitrogen and phosphorus at the land-sea margin of the North Atlantic Ocean." *Biogeochemistry*. 35(1), 141-180.

Pilson, M. E. Q. (1998). *An Introduction to the Chemistry of the Sea*. Prentice-Hall, Inc.

Ravikumar, S., Kathiresan, K., Thadedus Maria Ignatiammal, S., Babu Selvam, M. and Shanthy, S. (2004) "Nitrogen-fixing azotobacters from mangrove habitat and their utility as marine biofertilizers." *Journal of Experimental Marine Biology and Ecology*. 312, 5 - 17.

Ritchie, G. A. F., Nicholas, D. J. D. (1972) "Identification of the source of nitrous oxide produced by oxidative and reductive processes in *Nitrosomonas europaea*." *Biochemical Journal*. 126, 1181-1191.

Rosswall, T. (1983). *The Major Biogeochemical Cycles and Their Interactions*. (B. a. C. Bolin, R. B.), John Wiley & Sons, Inc.

Seitzinger, S., Nixon, S., Pilson, M. E. Q., and Burke, S. (1980) "Denitrification and N_2O production in near-shore marine sediments." *Geochimica et Cosmochimica Acta*. 44, 1853-1860.

Seitzinger, S. P., Giblin, A. E. (1996) "Estimating denitrification in North Atlantic continental shelf sediments." *Biogeochemistry*. 35, 235-260.

Seitzinger, S. P., Pilson, M. E. Q., and Nixon, S. W. (1983) "Nitrous oxide production nearshore marine sediments." *Science*. 222(4629), 1244-1246.

Sisler, F. D. a. Z., C. E. (1951) "Nitrogen fixation by sulfate-reducing bateria indicated by nitrogen/argon ratios." *Science*. 113(2940), 511-512.

Sorensen, J., Jorgensen, K. S., Colley, S., Hydes, D. J., Thomson, J., and Wilson, T. R. S. (1987) "The potential contribution of primary production by red tides to the west Florida shelf ecosystem." *Limnology and Oceanography*. 32, 762-767.

Staal, M., Hekkert, S. T., Brummer, G. J., Veldhuis, M., Sikkens, C., Persijn, S., Stal, L. J. (2007) "Nitrogen fixation along a north-south transect in the eastern Atlantic Ocean." *Limnology and Oceanography*. 52(4), 1305-1316.

Steppe, T. F. a. P., H.W. (2002) "Potential N_2 fixation by sulfate-reducing bacteria in a marine intertidal microbial mat." *Aquatic Microbial Ecology*. 28, 1-12.

Strous, M., Kuenen, J. G., and Jetten, M. S. M. (1999) "Key physiology of anaerobic ammonium oxidation." *Applied and Environmental Microbiology*. 65, 3248-3250.

van Luijn, F., Boers, P. C., Lijklema, L. and Sweerts, J.-P. R. A. (1999) "Nitrogen fluxes and processes in sandy and muddy sediments from a shallow eutrophic lake." *Water Research*. 33(1), 33-42.

Vidal, M., Duarte, C., Granata, T., Agusti, S. (1999) "Dissolved organic nitrigen and phosphorus pools and fluxes in the central Atlantic ocean." *Limnology and Oceanography*. 44, 106-115.

Yoshida, T., Alexander, M. (1970) "Nitrous oxide formation by *Nitrosomonas europaea* and heterotrophic microorganisms." *Soil Science Society of America Proceedings*. 34, 880-882.

CHAPTER 2

DENITRIFICATION PATHWAYS AND RATES IN SANDY SEDIMENTS OF THE GEORGIA CONTINENTAL SHELF, USA

2.1 Abstract

Denitrification in continental shelf sediments has been estimated to be a significant sink of oceanic fixed nitrogen (N). The significance and mechanisms of denitrification in organic-poor sands, which comprise 70% of continental shelf sediments, are not well known. Core incubations and isotope tracer techniques were employed to determine processes and rates of denitrification in the coarse-grained, sandy sediments of the Georgia continental shelf. In these sediments, heterotrophic denitrification was the dominant process for fixed N removal. Processes such as coupled nitrification-denitrification, anammox (anaerobic ammonium oxidation), and OLAND (oxygen-limited autotrophic nitrification-denitrification) were not evident over the 24- and 48-hour timescale of the incubation experiments. Heterotrophic denitrification processes produce 22.8 – 34.1 μmole N m^{-2} d^{-1} of N_2 in these coarse-grained sediments. These denitrification rates are approximately two orders of magnitude lower than rates determined in fine-grained shelf sediments. These lower rates may help reconcile unbalanced marine N budgets which calculate global N losses exceeding N inputs.

2.2 Introduction

Recent studies have reported imbalances in global marine fixed N budgets with rates of N loss exceeding rates of N input (Codispoti, 1995; Middelburg, 1996; Codispoti, 2001). These imbalanced budgets reflect the difficulties in making estimations given the many uncertainties in the pathways and rates of key N supply and removal reactions. In particular, the relatively few measurements of pathways and rates of denitrification reactions, the largest sink term in the global N budget, confound estimations of global N losses. Denitrification in continental shelf sediments is one of the largest sinks of oceanic N (Devol, 1991; Middelburg, 1996; Pilson, 1998), accounting for up to 67% of estimates of total global denitrification (Codispoti, 2001). Most direct denitrification rate measurements for continental shelves have been made on fine-grained, muddy sediments which cover only 30% of global shelf area (Emery, 1968). The remaining 70% of continental shelf area is covered by sandy sediments. These sandy sediment environments are generally characterized by low organic matter and high pore water dissolved oxygen concentrations, properties typically considered unfavorable for heterotrophic denitrification. The possibility of alternative pathways to N_2, which may not be limited by organic matter content, oxygen, or observed dissolved inorganic N levels, have not been examined in these widespread environments. This study of denitrification in the coarse-grained, sandy sediments of the Georgia continental shelf provides new information in an often overlooked but potentially significant sediment type.

Heterotrophic denitrification is the process of organic matter oxidation using nitrate (NO_3^-) and/or nitrite (NO_2^-) as electron acceptors (equation 2.1). Denitrifying

organisms are able to use oxygen as an electron acceptor under aerobic conditions and can switch to the denitrification pathway of organic matter oxidation under suboxic or anoxic conditions. The sandy sediments of the Georgia continental shelf contain very little organic matter, low NO_3^- and NO_2^- concentrations, and are overlain by an oxic water column (Hopkinson, 1991; Marinelli, 1998). Dissolved O_2 also penetrates these sediments to variable depths, depending on the season, photosynthetic activity (Marinelli, 1998), and light levels at the sea floor (Nelson, 1999) and can be circulated throughout the sediment by physical processes related to the interaction of bottom currents and the sediment surface (Huettel, 2000). Typically, these sediments would not be considered an ideal environment for denitrification. Under denitrifying conditions, NO_3^- concentrations would typically decrease with time as a reactant during denitrification (equation 2.1). Based on their observations of constant NO_3^- concentrations over time, Marinelli et al. (1998) suggested that denitrification was not occurring in sediments from the South Atlantic Bight, in which the Georgia continental shelf is located.

$$5CH_2O_{(organicmatter)} + 4NO_3^- + H^+ \rightarrow 2N_2 + 5CO_2 + 7H_2O \quad (2.1)$$

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+ \quad (2.2)$$

The coupled nitrification-dentrification mechanism has been suggested as an alternative pathway to the classical, heterotrophic denitrification process (Jenkins, 1984). Ammonium (NH_4^+) is oxidized to NO_2^- and/or NO_3^- during nitrification (equation 2.2), and those products can subsequently be reduced to N_2 during denitrification (equation 2.1). This coupled process must occur in different zones within the sediment because nitrification is an aerobic process and denitrification is an anaerobic process. Coupled nitrification-denitrification has been used to explain the occurrence of fixed N removal in

oxic environments (Hartnett, 2003). It can also be used to explain constant or even increasing NO_3^- concentrations in regions where denitrification is thought to be occurring (Codispoti, 1985; Laursen, 2002).

Other alternative pathways to heterotrophic denitrification that lead to the production of N_2 have been identified, increasing the difficulty in delineating the processes responsible for fixed N removal in natural systems. NH_4^+ and NO_2^- can react directly in the absence of organic matter to form N_2 in the reaction known as anammox (anaerobic ammonium oxidation) (equation 2.3). Anammox has been found to occur in natural systems (Dalsgaard, 2003; Kuypers, 2003) as well as in the wastewater reactor environment, where the reaction was discovered (Mulder, 1995; Strous, 1999; Kuenen, 2001). NH_4^+ can also be aerobically oxidized to form N_2 in the absence of organic matter in a two-step reaction termed OLAND (oxygen-limited autotrophic nitrification-denitrification) (Kuai, 1998; Verstraete, 1998) (equations 2.4a and 2.4b). The second step of the OLAND reaction (equation 2.4b) differs from the anammox reaction (equation 2.3) in that the NO_2^- that is consumed is produced during the first step of the reaction (Kuai, 1998), whereas anammox consumes ambient NO_2^- and/or NO_2^- produced as an intermediate of heterotrophic denitrification (Mulder, 1995; Dalsgaard, 2003; Kuypers, 2003). Anammox also differs from all of the other processes because it occurs within the cells of the anammox organisms, specifically in the anammoxosome, a special compartment where the reaction takes place (Sinninghe Damste, 2002). The other processes are known to be extracellular.

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad (2.3)$$

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+ \qquad (2.4a)$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad (2.4b)$$

Marine fixed N can be further removed through the anaerobic oxidation of ammonium by manganese dioxide (MnO_2) via two pathways:

$$4MnO_2 + NH_4^+ + 6H^+ \rightarrow 4Mn^{2+} + NO_3^- + 5H_2O \qquad (2.5a)$$

$$3MnO_2 + 2NH_4^+ + 4H^+ \rightarrow 3Mn^{2+} + N_2 + 6H_2O \qquad (2.5b)$$

Reaction (2.5a) indirectly removes fixed N by converting NH_4^+ to NO_3^-, which can be subsequently denitrified to form N_2. The Mn cycle interacts with the N cycle through yet another reaction involving the reduction of nitrate by Mn^{2+}:

$$5Mn^{2+} + 2NO_3^- + 4H_2O \rightarrow 5MnO_{2(solid)} + N_2 + 8H^+ \qquad (2.5c)$$

The coupling of the manganese cycle to the nitrogen cycle has been cited as an alternative means of fixed N removal and N_2 production (Sorensen, 1987; Aller, 1990; Murray, 1995; Luther, 1997; Hulth, 1999). These reactions were found to occur in fine-grained, deep, continental margin sediments (Luther, 1997). Although it is important to recognize their potential influence on the N cycle, specific examination of these reactions is beyond the scope of this study.

Beyond discerning the different denitrification pathways, the influence of organic matter quantity and benthic primary production on heterotrophic denitrification is also not clear. Because organic matter is consumed during heterotrophic denitrification, it must be present in order for the reaction to occur. Sandy sediments have been overlooked in some denitrification studies due to their typically low organic carbon (C) content. It has been proposed that the bioreactivity of organic matter is more important than the quantity of organic matter for heterotrophic denitrification (Laursen, 2002; Hartnett, 2003). Benthic primary production may be able to supply the bioavailable

organic matter required to support heterotrophic denitrification in these sediments. However, the utilization of nutrients, such as NH_4^+, by these benthic organisms may effectively block their regeneration to the overlying water, thus limiting processes such as nitrification and denitrification (Marinelli, 1998; An, 2001). Also, oxygen produced during benthic primary production (Jahnke, 2000) may influence nitrification (equation 2.2) (An, 2001) and OLAND (equation 2.4a) (Kuai, 1998; Verstraete, 1998) reactions. Hence, it is unclear what the net effect of benthic primary production would be on denitrification.

Due to the many reactions that may produce or consume nitrite, nitrate, and ammonium, measurements of N_2 provide the most direct evidence of denitrification (Devol, 1991; Kana, 1994; Mulder, 1995; An, 2001). Typically, it is difficult to measure small changes in dissolved N_2 relative to the high ambient concentrations in seawater. Because the sedimentary characteristics of Georgia shelf sediments are not typically conducive for denitrification, it is necessary to employ techniques sensitive enough to detect relatively low denitrification rates. Isotope tracer techniques using ^{15}N-labelled NO_3^- and NH_4^+ during sediment incubation experiments can yield various isotopically heavy N_2 gases depending on the N cycling mechanisms involved. The isotopically heavy N_2 gases produced during incubations are easily observed relative to their low ambient concentrations in seawater (Kokkinakis, 1987; Nielsen, 1992) and can be measured using membrane inlet mass spectrometry. Sediment cores were collected from the Georgia continental shelf and incubated in the presence of labeled NO_3^- or NH_4^+ to determine the presence of denitrification, and if present, the pathways and rates to N_2.

2.3 Methods

2.3.1 Sampling

Measurements of denitrification rates as well as other processes have been limited in sandy sediments because of the difficulty in collecting cores and retaining the pore-water. Sediment cores were collected using a modified spade corer with a metal sleeve designed to fit cylindrical core barrels, which replaced the core box. The acrylic core barrels used in this method (7cm diameter x 34 cm length x 0.65 cm wall) are fitted with a polyvinyl chloride annulus at the top of the barrel, consisting of two O-rings on the outer surface of the annulus and one O-ring on the inner surface. During retrieval of the sediment core and overlying water, a solid acrylic ball rests upon the O-ring that is placed on the inner surface of the annulus, creating a seal and preventing pore-water leakage. Upon return of the corer from the bottom of the sea floor, a bottom piston containing two O-rings is inserted into the core barrel, the ball-valve system is removed from the top, and the overlying water of the core is open to the atmosphere (Marinelli, 1998).

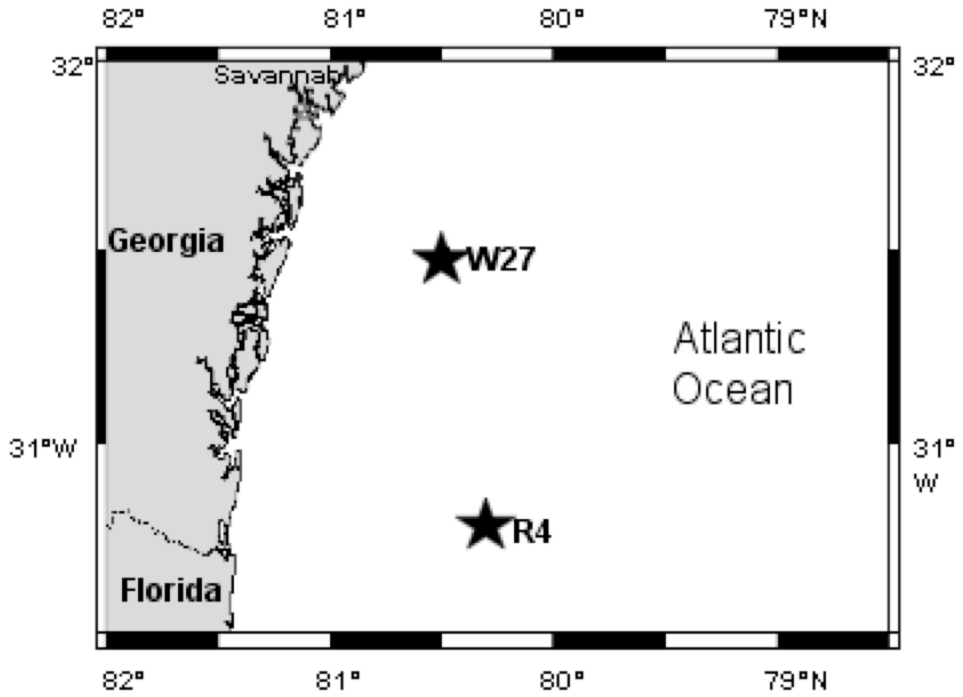

Figure 2.1: Sampling Sites. Cores were collected from W27 site in July 2002. W27 is along the Wassaw transect and has a depth of 27m. The Wassaw transect was characterized by Marinelli et al. Cores were also collected from the R4 Tower site in April 2004, which is among eight platforms that are used to collect oceanographic and meteorological data for the South Atlantic Bight Synoptic Offshore Observational Network (SABSOON). This site is located at a depth of 40m.

2.3.1.1 W27 Cores

Two sediment cores and bottom water samples were collected on July 23, 2002, at W27 site located on the Georgia continental shelf at a depth of 27m in the South Atlantic Bight (Figure 2.1). The cores contained 15.5 cm of sediment with 202 mL of overlying water. The overlying water of these cores contained 1μM NO_3^-, no detectable NO_2^-, and 6-9μM

NH_4^+. This site was characterized in a study conducted by Marinelli et al. (1998), which analyzed the sediment biogeochemistry along a transect in the South Atlantic Bight. The porosity of the sediment at all sites is roughly 0.5 (Marinelli, 1998). The range of O_2 penetration into the sediments is between 6 and 9 cm during the summer months (Marinelli, 1998). The incubation experiment conducted on these cores (see section 2.3.3.1) was performed on the *R/V Savannah,* and samples were collected and stored on ice during transport back to the Georgia Institute of Technology in Atlanta, GA.

2.3.1.2 R4 Cores

Six sediment cores and bottom water samples were collected on April 23, 2004, at R4 Tower, also located on the Georgia continental shelf at a depth of 40m in the South Atlantic Bight (Figure 2.1). These cores contained between 15 – 18 cm of sediment and 190 – 215 mL of overlying water. The R4 Tower is one of eight platforms in the South Atlantic Bight off of the Southeastern U.S. The platforms are a part of the South Atlantic Bight Synoptic Offshore Observational Network (SABSOON), which is used to obtain continuous and real-time oceanographic and meteorological data in this region. The core samples were transported back to the Georgia Institute of Technology and stored in an environmental chamber at bottom water temperature (18°C).

2.3.2 Isotope tracer technique

The natural abundance of the isotope ^{14}N is 99.634%. When denitrification occurs (equation 2.1), the dominant product is $^{28}N_2$. Due to the naturally high concentration of dissolved N_2 in seawater, it is often difficult to observe the small additions of $^{28}N_2$ from denitrification. The other stable isotope of N, ^{15}N, has a natural abundance of 0.366%. Using the isotope tracer technique, nitrate strongly enriched (>98%) in the ^{15}N isotope ($^{15}NO_3^-$) is used as a reactant during denitrification and can react with ambient $^{14}NO_3^-$ molecules to produce $^{29}N_2$. This tracer can also react with other $^{15}NO_3^-$ molecules to produce $^{30}N_2$. The production of these two isotopically distinct end products is much higher than would be observed during natural denitrification.

NH_4^+ is used as a substrate in some of the alternative pathways to N_2 such as coupled nitrification-denitrification, anammox, and OLAND. In order to detect the presence of these alternative pathways via the production of $^{29}N_2$ and $^{30}N_2$, ammonium enriched (>98%) in the ^{15}N isotope ($^{15}NH_4^+$) can also be used as a tracer. The schematic shown in figure 2.2 shows the potential combination of N isotopes in N_2 gas that would be produced as a result of different N cycling processes during the incubation experiments.

2.3.3 Incubation Experiment

2.3.3.1 W27 cores

In order to detect the presence of denitrification in the sandy sediments of the Georgia continental shelf, an isotope tracer experiment was conducted on intact cores collected from the W27 site in the dark. The overlying water of one core was replaced with an amendment solution containing 50 μM $Na^{15}NO_3$ (>98% ^{15}N), which was made using the bottom water collected from the site. The other core was not amended and was used as a control. A syringe was inserted into self-sealing rubber septa on the core barrels to collect samples of the overlying water (Marinelli, 1998) of both cores at the start of the experiment and after 24 hours. The samples were stored under ice water before dissolved gas analyses.

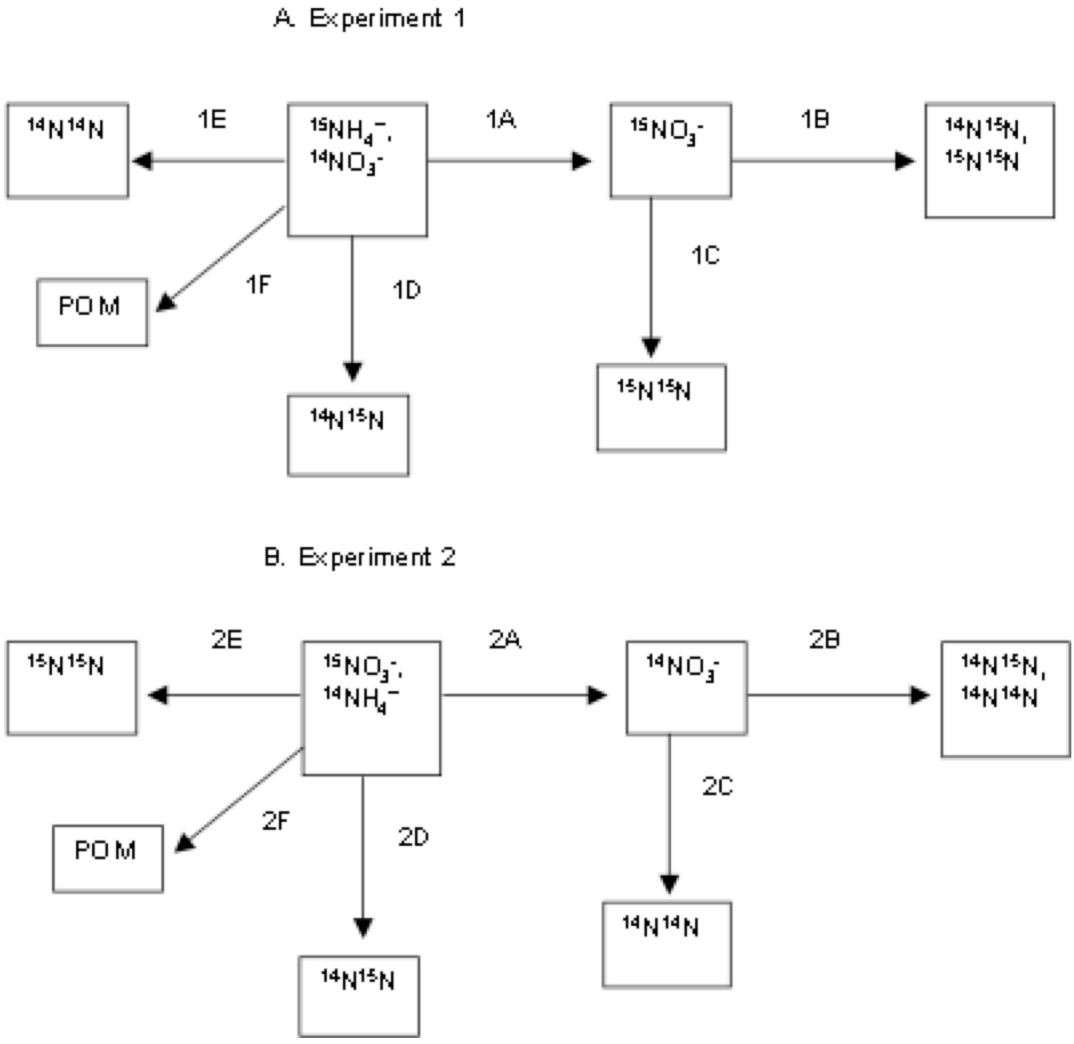

Figure 2.2: Possible outcomes of amendment experiments. 1A = Aerobic nitrification of $^{15}NH_4^+$; 1B = Heterotrophic denitrification with $^{14}NO_3^-$ and/or $^{15}NO_3^-$; 1C = OLAND with $^{15}NH_4^+$ or partial nitrate reduction to nitrite followed by anammox with $^{15}NH_4^+$; 1D = Same as 1C except with standard nitrate; 1E = Heterotrophic denitrification with standard nitrate; 1F = Assimilation. 2A = Aerobic nitrification of standard ammonium; 2B = Heterotrophic denitrification with $^{14}NO_3^-$ and/or $^{15}NO_3^-$; 2C = OLAND with standard ammonium or partial nitrate reduction to nitrite followed by anammox with standard ammonium; 2D = Same as 2C except with $^{15}NO_3^-$; 2E = Heterotrophic denitrification of $^{15}NO_3^-$; 1F = Assimilation

2.3.3.2 R4 cores

Two different incubation experiments were conducted on intact cores to detect the denitrification pathways in the sandy sediments of the Georgia continental shelf. All experiments were done in the dark. In order to make a comparison between these experiments and the experiment on W27 cores, 50µM solutions were also used. In experiment 1, the overlying water in two cores was replaced with a solution containing 50µM $^{15}NH_4Cl$ (>98% ^{15}N) and 50µM $NaNO_3$ using the bottom water from the sampling site. Similarly, in experiment 2, the overlying water of two different cores was replaced with a solution containing 50 µM $Na^{15}NO_3$ and 50µM NH_4Cl. The two remaining cores were used as controls. After replacing the overlying water of the cores, they were sealed with a piston. After 24 and 48 hours, the overlying water was sampled by pressing the top pistons down and allowing the water to flow out of a hole that was drilled into the piston and into a long, glass sampling tube. Sampling techniques were designed to minimize exchange of atmospheric N_2 with solution. The samples were then capped and stored under ice water for dissolved gas analyses using membrane inlet mass spectrometry.

2.3.3.3 Core incubation vs. in-situ benthic landers

Hammond et al. (2004) recently addressed two common techniques used to study nutrient fluxes: in situ benthic landers and core incubations. Overall, the core incubation technique provided similar results to the in situ lander results. The core incubation technique, however was found to have some minor drawbacks. During the incubation experiment nitrate uptake was observed to be ~34% lower than the observed in situ rates.

They concluded that denitrification rates may be underestimated by incubation studies (Hammond, 2004). Given that the objective of this paper is to identify denitrification rates and pathways in an environment that is often overlooked, an underestimation of measured denitrification would suggest that the results are a lower limit of the actual denitrification rates in these sandy sediments.

2.3.4 Membrane Inlet Mass Spectrometry

Investigation of denitrification in all marine environments has been hampered by the complexity of measuring dissolved N_2 in natural waters. Previous investigations of denitrification rates have utilized a variety of techniques including stoichiometric calculations, gas chromatography, acetylene block, and in situ measurements using benthic chambers (Seitzinger, 1980; Devol, 1991; Blackburn, 1993; Devol, 1993; Christensen, 1996; Seitzinger, 1996; van Luijn, 1996; Marinelli, 1998; van Luijn, 1999; Laursen, 2002). Membrane inlet mass spectrometry, or MIMS, is another approach to dissolved gas measurement developed by Kana et al. (1994). Some advantages of MIMS include: no separate degassing step (a source of error in other methods), small sample size (8-10 mL), measurements can be made in a timely fashion (≈15 samples/hr), and high precision (<0.05% error for N_2/Ar analyses) (Kana, 1994). The instrument can detect masses 28, 29, and 30, which can be attributed to various combinations of the stable isotopes of N (^{14}N, ^{15}N) in N_2. Other gases with these masses are removed before analysis with a cryogenic trap. It has been noted that the ability of the MIMS to detect $^{30}N_2$ can be influenced by dissolved O_2 concentration (Eyre, 2002). O_2 can react with N_2 in the ion source of the mass spectrometer to form NO^+, which also has a mass of 30.

When calculating the concentration of $^{30}N_2$, this O_2 effect is taken into consideration using the approach described in Eyre et al. (2002). Further information on the MIMS technique can be obtained in Kana et al. (1994).

2.4 Results and Discussion

2.4.1 Heterotrophic Denitrification and Alternative Pathways

2.4.1.1 W27 cores

MIMS analyses on samples obtained from the core amended with 50 μM $^{15}NO_3^-$ showed increases in both $^{29}N_2$ and $^{30}N_2$, while the control samples did not show an increase in either of the dissolved gases (Figure 2.3). Because $^{15}NO_3^-$ was the only significant source of ^{15}N-N, it is clear that the production of $^{29}N_2$ and $^{30}N_2$ was the result of the reduction of the $^{15}NO_3^-$ tracer. These results indicate that some form of denitrification occurs in Georgia continental shelf sediments.

2.4.1.2 R4 cores

Experiment 1 used a solution amended with $^{15}NH_4^+$ and standard NO_3^-. MIMS gas analyses on samples from this experiment, obtained 24 and 48 hours after the start of the incubation, showed no increase in $^{30}N_2$ or $^{29}N_2$ (Figure 2.3). According to figure 2, the absence of detectable $^{29}N_2$ and $^{30}N_2$ indicates that alternative pathways such as coupled nitrification-denitrification, anammox, and OLAND are not significant on the timescale of these experiments.

Experiment 2 used a solution amended with $^{15}NO_3^-$ and standard NH_4^+. MIMS gas analyses on these samples after 48 hours showed an increase in both $^{29}N_2$ and $^{30}N_2$ (Figure 2.3). As seen in figure 2, the only source of ^{15}N was the $^{15}NO_3^-$ tracer, thus the increase in $^{30}N_2$ must be due to heterotrophic denitrification. Also, according to figure 2.2, the increase in $^{29}N_2$ can be

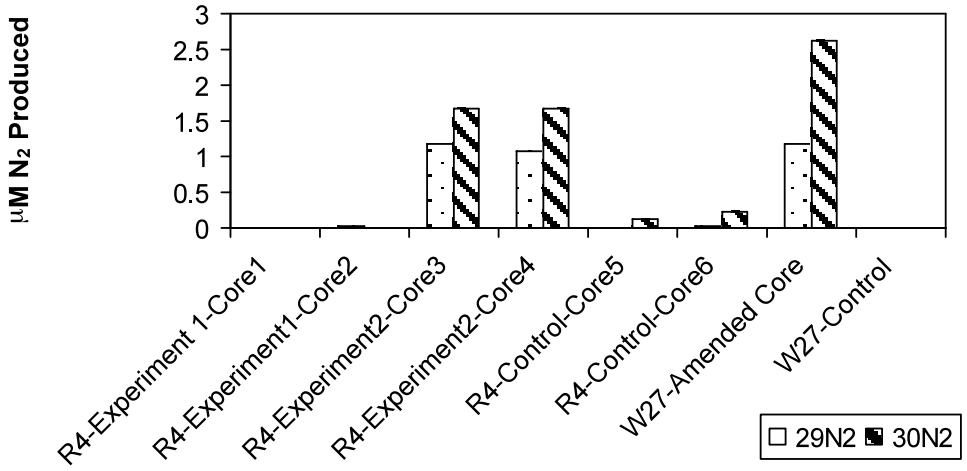

Figure 2.3: Concentrations of dissolved $^{29}N_2$ and $^{30}N_2$ in core incubations after 48 hours in R4 cores and 24 hours in W27 cores. R4-Experiment 1 was amended with $^{15}NH_4^+$ and standard NO_3^-. R4-Experiment 2 was amended with $^{15}NO_3^-$ and standard NH_4^+. W27-Amended core was amended with $^{15}NO_3^-$. The omission of a bar indicates that the production of that gas was negligible.

explained by a number of possibilities such as the presence of OLAND or anammox reactions or the coupled nitrification-denitrification mechanism. However, those possibilities can be eliminated due to the lack of evidence for an alternative pathway to N_2 as observed in experiment 1. Therefore, the increase in $^{29}N_2$ suggests that a fraction of the ambient $^{14}NO_3^-$ was denitrified with the $^{15}NO_3^-$ tracer to produce the heavy gas.

The fraction of ambient $^{14}NO_3^-$ denitrified with the $^{15}NO_3^-$ tracer can be estimated. Given the area, depth, and volume of water (including porewater) of the cores, and the porosity of the sediment, the average amount of ^{14}N and ^{15}N was calculated for W27 and R4-experiment 2 as 1.1 and 10.4 μmol, respectively. The N in the system was therefore composed of 10% ^{14}N and 90% ^{15}N. Using the amount of $^{29}N_2$ and $^{30}N_2$ that was produced in the cores (Figure 2.3), it was estimated that 48-53% of the total ^{14}N in the system was used to produce $^{29}N_2$ and 37-56% of the total ^{15}N in the system was used to produce both $^{29}N_2$ and $^{30}N_2$. These calculations suggest that the $^{29}N_2$ produced during the two experiments could have been the result of heterotrophic denitrification of ambient $^{14}NO_3^-$ with the $^{15}NO_3^-$ tracer.

The sandy sediments of the Georgia continental shelf contain very little organic C (0.03 – 0.12%) in comparison to typical continental shelf sediments (0.7%) (Nelson, 1999). The presence of heterotrophic denitrification in these sediments suggests that organic matter quantity is not a good indication of the potential of a particular sediment to support denitrification. Benthic primary production in these sediments is significant and comparable to water column primary production (Jahnke, 2000), thus providing a source of fresh, bioavailable organic matter, which may be easily oxidized during heterotrophic denitrification. Although the influence that benthic organisms have on heterotrophic denitrification with regards to O_2 and nutrient dynamics was not directly measured as a part of the current study, the results indicate that benthic primary production has a net positive effect in supporting heterotrophic denitrification in Georgia shelf sands.

2.4.2 Denitrification Rates

The concentrations of $^{29}N_2$ and $^{30}N_2$ measured in samples from W27 and R4-experiment 2 were used to obtain the rate of heterotrophic denitrification in these sediments. The gas production fluxes were calculated using the following equation:

$$r = \frac{([N_2]_f - [N_2]_i)}{A * t} * (V_{ow} + \phi * V_{sed}) \qquad (2.6)$$

where $[N_2]_f$ = final concentration of N_2 gas in the overlying water of the core, $[N_2]_i$ = initial concentration of N_2 gas in the overlying water, A = area of sediment, t = length of time of the experiment, V_{ow} = volume of overlying water, V_{sed} = volume of sediment, ϕ = porosity of the sediment. To estimate ambient denitrification rates based on the gas fluxes obtained in isotopically amended core incubations, the method of Nielsen was used (Nielsen, 1992). Denitrification estimates based on these calculations yield a range of 22.8 – 34.1 μmol N m^{-2} d^{-1} for Georgia continental shelf sediments. Also, the rates obtained from the W27 experiment (34.1 μmole N m^{-2} d^{-1}), which used an amendment solution of 50μM $^{15}NO_3^-$, are slightly higher than those obtained from R4-Experiment 2 (22.8 & 23.2 μmole N m^{-2} d^{-1}), which used a solution of 50μM $^{15}NO_3^-$ and 50μM standard NH_4^+. If an alternative denitrification pathway existed in these sediments, the rates obtained from R4-experiment 2 should have been higher than those obtained in the W27 experiment, which was not observed. These results further suggest that the $^{29}N_2$ that was produced during R4-experiment 2 was a result of ambient $^{14}NO_3^-$ being denitrified with the tracer $^{15}NO_3^-$.

2.4.3 Comparison to literature

In a study conducted by Laursen and Seitzinger (2002), denitrification rates in various continental shelf sediments were reported from other studies. Laursen and Seitzinger, (2002) using the dissolved inorganic N concentrations and N:P ratios determined by Hopkinson et al. (1991) for Georgia Bight sediments, obtained an average denitrification rate of 3200 μmol N m^{-2} d^{-1} for the Georgia Bight (compared to 22.8 – 34.1 μmol N m^{-2} d^{-1} from this study). However, this data was collected from Gray's Reef, a site in the Georgia Bight that has a unique benthic organism community and contains a heterogeneous sediment type throughout the site (Hopkinson, 1991). Gary's Reef is a hard bottom habitat, which consists of biological assemblages of a variety of organisms that are attached to hard surfaces on the sea floor. There are regions within the reef of high epifaunal biomass, which correlated with high nutrient regeneration rates. Organic C levels were also substantially higher in the sediments at Gray's Reef than in the surrounding sandy sediments (Hopkinson, 1991). The sampling location of this study is very different from the environment of Gray's Reef, which may contribute to the markedly different denitrification rates obtained in these two studies.

In comparison to the rates determined by this study, higher rates were also obtained by other studies of continental shelf sediments (700-3200 μmol N m^{-2} d^{-1}) (Devol, 1993; Seitzinger, 1996; Laursen, 2002). These lower rates are not surprising when considering the differences between the sedimentary characteristics of the Georgia continental shelf and those of the continental shelves in which the above denitrification rates were measured. These other study sites contain fine-grained, muddy, organic-rich sediments as opposed to the coarse-grained, sandy, organic-poor sediments of the

Georgia continental shelf. Although Georgia Bight sediments are receiving a fresh supply of reactive organic matter from benthic primary production, there is still very little organic matter (0.03% - 0.12% organic C by weight compared to 0.7% for typical shelf sediments (Nelson, 1999)) to be oxidized during heterotrophic denitrification. The O_2 present in these sediments may also have some inhibitory effect on denitrification rates because heterotrophic denitrification is typically an anaerobic process.

The marine fixed nitrogen budget has been the subject of much controversy over the past 20 years. Some studies have shown that the N budget is balanced and that the ocean is in steady-state with respect to N (Codispoti, 1985; Gruber, 1997). Other studies have shown that fixed N is being removed from the ocean at a higher rate than it is being supplied, suggesting that the ocean is losing fixed N (Codispoti, 1995; Middelburg, 1996; Codispoti, 2001). One of the sources of this ongoing debate is the uncertainty in many of the denitrification estimates that have been made. Direct measurements of denitrification have been made at relatively few locations throughout the world's oceans (Brandes, 2002). The global budgets that have been proposed are dependent upon both direct and indirect measurements of denitrification that have been extrapolated to larger regions (Middelburg, 1996; Seitzinger, 1996).

2.5 Conclusions

The data obtained during this study suggest that heterotrophic denitrification, occurring at a rate of 22.8 – 34.1 μmol N m^{-2} d^{-1}, is the only pathway to N_2 in Georgia shelf sediments. These direct estimates of denitrification rates, if representative of coarse-grained, sandy sediments worldwide, would lower the denitrification sink term for fixed

N in continental shelf sediments. These results show that N_2 produced from isotope tracers at relatively low rates can be detected by MIMS on reasonable experimental time scales. Further studies of the Georgia continental shelf examining potential spatial and temporal trends as well as the influence of benthic primary production on denitrification are necessary. A better-constrained range of denitrification rates in these sediments will allow this region and perhaps other regions dominated by coarse-grained, sandy sediments to be better represented in global N budgets.

2.6 References

Aller, R. C. (1990) "Bioturbation and manganese cycling in hemipelagic sediments." Philosophical Transactions of the Royal Society of London Series B - Biological Sciences. 331, 51-68.

An, S., Gardner, W. S. and Kana, T. (2001) "Simultaneous Measurement of denitrification and nitrogen fixation using isotope pairing with membrane inlet mass spectrometry analysis." Applied and Environmental Microbiology. 67(3), 1171-1178.

An, S., Joye, S. B. (2001) "Enhancement of coupled nitrification-denitrification by benthic photosynthesis in shallow estuarine sediments." Limnology and Oceanography. 46(1), 62-74.

Blackburn, N. D., Blackburn, T.H. (1993) "A reaction diffusion model of C-N-S-O species in a stratified sediment." FEMS Microbiology Ecology. 102, 207-215.

Brandes, J., Devol, A.,. (2002) "A global marine-fixed nitrogen isotopic budget: Implications for Holocene nitrigen cycling." Global Biogeochemical Cycles. 16, 67.1-67.14.

Christensen, J. P., Townsend, D. W. and Montoya, J. P. (1996) "Water column nutrients and sedimentary denitrification in the Gulf of Maine." Continental Shelf Research. 16(4), 489-515.

Codispoti, L. A. (1995) "Is the ocean losing nitrate?" Nature. 376, 724.

Codispoti, L. A., Brandes, J. A., Christensen, J. P., Devol, A. H., Naqvi, S. W. A., Paerl, H. W., and Yoshinari, T. (2001) "The oceanic fixed nitrogen and nitrous oxide

budgets: Moving targets as we enter the anthropocene?" Scientia Marina. 65, 85-105.

Codispoti, L. A., Christensen, J. P. (1985) "Nitrification, denitrification, and nitrous oxide cycling in the eastern tropical South Pacific Ocean." Marine Chemistry. 16, 277-300.

Dalsgaard, T., Canfield, D. E., Petersen, J., Thamdrup, B., Acuna-Gonzalez, J. (2003) "N_2 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica." Nature. 422, 606-608.

Devol, A. H. (1991) "Direct measurement of nitrogen gas fluxes from continental shelf sediments." Nature. 349(6307), 319-321.

Devol, A. H., Christensen, J. P. (1993) "Benthic fluxes and nitrogen cycling in sediments of the continental margin of the eastern North Pacific." Journal of Marine Research. 51, 345-372.

Emery, K. O. (1968) "Relict sediments on continental shelves of the world." American Association of Petrology and Geology. 52, 445-464.

Eyre, B. D., Rysgaard, S., Dalsgaard, T., Christensen, P. B. (2002) "Comparison of Isotope Pairing and N2:Ar Methods for Measuring Sediment Denitrification-Assumptions, Modifications, and Implications." Estuarines. 25, 1077-1087.

Gruber, N., Sarmiento, J. L. (1997) "Global patterns of marine nitrogen fixation and denitrification." Global Biogeochemical Cycles. 11, 235-266.

Hammond, D. E., Cummins, K. M., McManus, J., Berelson, W. M., Smith, G., and Spagnoli, F. (2004) "A comparison of methods for benthic flux measurement along the California margin: Shipboard core incubations vs. in situ benthic landers." Limnology and Oceanography: Methods. 2, 146-159.

Hartnett, H. E., Devol, A.H. (2003) "Role of a strong oxygen-deficient zone in the preservation and degradation of organic matter: A carbon budget for the continental margins of northwest Mexico and Washington State." Geochimica et Cosmochimica Acta. 67(2), 247-264.

Hopkinson, C. S., Fallon, R. D., Jansson, B-O, Schubauer, J. P. (1991) "Community metabolism and nutrient cycling at Gray's Reef, a hard bottom habitat in the Georgia Bight." Marine Ecology Progess Series. 73, 105-120.

Huettel, M., Rusch, A. (2000) "Transport and degradation of phytoplankton in permeable sediment." Limnology and Oceanography. 45, 534-549.

Hulth, S., Aller, R. C., and Gilbert, F. (1999) "Coupled anoxic nitrification/manganese reduction in marine sediments." Geochimica et Cosmochimica Acta. 63(1), 49-66.

Jahnke, R. A., Nelson, J. R., Marinelli, R. L. and Eckman, J. E. (2000) "Benthic flux of biogenic elements on the Southeastern US continental shelf: influence of pore water advective transport and benthic microalgae." Continental Shelf Research. 20, 109-127.

Jenkins, M. C., Kemp, W. M. (1984) "The coupling of nitrification and denitrification in two estuarine sediments." Limnology and Oceanography. 29, 609-619.

Kana, T. M., Darkangelo, C., Hunt, M. D., Oldham, J. B., Bennett, G. E. and Cornwell, J. C. (1994) "Membrane inlet mass spectrometry for rapid high-precision determination of N_2, O_2, and Ar in environmental water samples." Analytical Chemistry. 66(23), 4166-4170.

Kokkinakis, S. A., Wheeler, P. A. (1987) "Nitrogen uptake and phytoplankton growth in coastal upwelling regions." Limnology and Oceanography. 32(5), 1112-1123.

Kuai, L., Verstraete, W. (1998) "Ammonium Removal by the Oxygen-Limited Autotrophic Nitrification-Denitrification System." Applied and Environmental Microbiology. 64, 4500-4506.

Kuenen, J. G., Jetten, M. S. M. (2001) "Extraordinary anaerobic ammonium-oxidizing bacteria." American Society for Microbiology News. 67, 456-463.

Kuypers, M. M. M., Sliekers, A. O., Lavik, G., Schmid, M., Jorgensen, B. B., Kuenen, J. G., Damste, J. S. S., Strous, M., Jetten, M. S. M. (2003) "Anaerobic ammonium oxidation by anammox bacteria in the Black Sea." Nature. 422, 608-611.

Laursen, A. E., Seitzinger, S. P. (2002) "The role of denitrification in nitrogen removal and carbon mineralization in Mid-Atlantic Bight sediments." Continental Shelf Research. 22, 1397-1416.

Luther, G. W., Sundby, B., Lewis, B. L., Brendel, P. J., and Silverberg, N. (1997) "Interactions of manganese with the nitrogen cycle: Alternative pathways to dinitrogen." Geochimica et Cosmochimica Acta. 61(19), 4043-4052.

Marinelli, R. L., Jahnke, R.A., Craven, D.B., Nelson, J.R., and Eckman, J.E. (1998) "Sediment nutrient dynamics on the South Atlantic Bight continental shelf." Limnology and Oceanography. 43(6), 1305-1320.

Middelburg, J. J., Soetaert, K., Herman, P. M. J., and Heip, C. H. R. (1996) "Denitrification in marine sediments: A model study." Global Biogeochemical Cycles. 10(4), 661-673.

Mulder, A., van de Graaf, A. A., Robertson, L. A., and Kuenen, J. G. (1995) "Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor." FEMS Microbiology Ecology. 16, 177-184.

Murray, J. W., Codispoti, L. A., and Friederich, G. E. (1995) The suboxic zone in the Black Sea. Aquatic Chemistry: Interfacial and INterspecies Processes. (C. P. Huang, O'Melia, C. R., and Morgan, J. J.): 157-176, American Chemical Society.

Nelson, J. R., Eckman, J. E., Robertson, C. Y., Marinelli, R. L., and Jahnke, R. A. (1999) "Benthic microalgal biomass and irradiance at the sea floor on the continental shelf of the South Atlantic Bight: Spatial and temporal variability and storm effects." Continental Shelf Research. 19, 477-505.

Nielsen, L. P. (1992) "Denitrification in sediment determined from nitrogen isotope pairing." FEMS Microbiology Ecology. 86, 357-362.

Pilson, M. E. Q. (1998). An Introduction to the Chemistry of the Sea. Prentice-Hall, Inc.

Seitzinger, S., Nixon, S., Pilson, M. E. Q., and Burke, S. (1980) "Denitrification and N_2O production in near-shore marine sediments." Geochimica et Cosmochimica Acta. 44, 1853-1860.

Seitzinger, S. P., Giblin, A. E. (1996) "Estimating denitrification in North Atlantic continental shelf sediments." Biogeochemistry. 35, 235-260.

Sinninghe Damste, J. S., Stous, M., Rijpstra, W. I. C., Hopmans, E. C., Geenevasen, J. A. J., van Duin, A. C. T., van Niftrik, L. A., and Jetten, M. S. M. (2002) "Lineraly concatenated cyclobutane lipids form a dense bacterial membrane." Nature. 419, 708-712.

Sorensen, J., Jorgensen, K. S., Colley, S., Hydes, D. J., Thomson, J., and Wilson, T. R. S. (1987) "The potential contribution of primary production by red tides to the west Florida shelf ecosystem." Limnology and Oceanography. 32, 762-767.

Strous, M., Kuenen, J. G., and Jetten, M. S. M. (1999) "Key physiology of anaerobic ammonium oxidation." Applied and Environmental Microbiology. 65, 3248-3250.

van Luijn, F., Boers, P. C. M. and Lijklema, L. (1996) "Comparison of denitrification rates in lake sediments obtained by the N_2 flux method, the ^{15}N isotope pairing technique and the mass balance approach." Water Research. 30(4), 893-900.

van Luijn, F., Boers, P. C., Lijklema, L. and Sweerts, J.-P. R. A. (1999) "Nitrogen fluxes and processes in sandy and muddy sediments from a shallow eutrophic lake." Water Research. 33(1), 33-42.

Verstraete, W., Philips, S. (1998) "Nitrification-denitrification processes and technologies in new contexts." Environmental Pollution. 102, 717-726.

CHAPTER 3

INSIGHTS INTO THE EFFECT OF DISSOLVED OXYGEN CONCENTRATIONS ON N_2 MEASUREMENTS USING MEMBRANE INLET MASS SPECTROMETRY: IMPLICATIONS FOR DENITRIFICATION MEASUREMENTS

3.1 Abstract

Membrane inlet mass spectrometry (MIMS) is a dissolved gas analyses technique that has become widely used for measurements of denitrification, the conversion of biologically available nitrogen to gaseous end products, N_2 and N_2O. In natural waters with relatively constant dissolved oxygen concentrations, MIMS has been demonstrated to be a reliable method for dissolved N_2 gas measurements. However, in systems with variable dissolved O_2 concentrations, measurements may be confounded by the reaction of N_2 with O^+, fragmented from O_2, in the ion source of the mass spectrometer to form nitric oxide, NO^+. This phenomenon, known as the O_2 effect, can lead to large errors in dissolved N_2 measurements. Literature studies indicate that the O_2 effect is instrument specific. In this study, the O_2 effect was investigated on a MIMS system and provides the first explanation for the differences between systems. Differences are related to oxygen and nitrogen number densities in the mass spectrometer source, which are, in turn, a function of solution flow rates through the membrane inlet. An alternative method for calculating $^{30}N_2$ concentrations was developed for samples containing a wide range of dissolved O_2 levels. This method was applied to two isotope tracer studies, resulting in lower estimates of denitrification and anammox. A flow rate of 1 mL/min was determined to be

optimal for MIMS measurements as to balance NO^+ production and the time to steady-state.

3.2 Introduction

Membrane inlet mass spectrometry (MIMS) is a gas analyses technique developed over the past 15 years to measure dissolved gases in aqueous environmental samples (Kana, 1994; An, 2001; Eyre, 2002; Tortell, 2005). In particular, MIMS has been especially useful in measuring dinitrogen (N_2) gas produced by denitrification (equation 3.1) reactions, a key component of the global N cycle. It has been suggested that fixed N is the limiting nutrient for primary productivity in the global ocean. Denitrification is the most significant removal process of marine fixed N. Understanding the nuances of a technique that is used to measure denitrification more accurately is vital to constraining the global N budget.

$$5CH_2O_{org.matter} + 4NO_3^- + 4H^+ \rightarrow 2N_2 + 5CO_2 + 7H_2O \qquad (3.1)$$

Membrane inlet mass spectrometry is advantageous as a dissolved gas measuring technique because of its ability to use small sample sizes, analyze samples quickly, and produce results with high precision (Kana, 1994; An, 2001; Thomas, 2001; Laursen, 2002; Vance-Harris, 2005; Rao, 2007). MIMS has been gaining popularity as an effective tool for measuring denitrification since the release of the groundbreaking paper by Kana et al. (Kana, 1994), in which it was used to determine N_2:Ar and O_2:Ar ratios, and ultimately N_2 concentrations, in water samples. Since then, there have been other studies that have significantly contributed to the development and understanding of membrane inlet mass spectrometry. These studies are in disagreement over the effect of dissolved

oxygen concentrations on the ability of the MIMS to accurately measure dissolved N_2 (Eyre, 2002; Kana, 2004; Eyre, 2004).

3.2.1 Membrane Inlet Mass Spectrometry (MIMS)

In the study by Eyre et al., 2002, the authors conducted experiments whereby the concentration of dissolved O_2 in seawater was varied over known concentrations, without changing the N_2 concentration. Although N_2 was held constant in these experiments, the results indicated apparent differences in N_2 concentrations. A linear relationship between the measured N_2 and O_2 signals in these experiments suggested a relation between these parameters. Mechanistically, this relation can be explained by a reaction between N_2 and O^+, fragmented from O_2, within the ion source of the mass spectrometer to produce NO^+. Consistent with the mechanism, their study showed that as O_2 concentrations increased, the MIMS output signal of N_2 decreased. The reverse effect of an increasing N_2 signal resulting from decreasing O_2 concentrations was also shown. This oxygen concentration dependent experimental artifact has serious implications for denitrification measurements using MIMS. Denitrification reactions typically occur in environments with strong gradients in redox conditions. In such environments, oxygen concentrations can vary widely.

Other studies have shown little or no relation between O_2 concentrations and N_2 measurements (Kana, 1994; Rao, 2007). The differences between studies indicate that the O_2 effect on N_2 measurements using MIMS may be instrument specific. However, there has been no mechanistic explanation for the observed differences between different instruments of nearly identical construction.

3.2.2 Experimental Approaches

In this paper, a series of experiments was performed to understand instrument-specific effects previously observed on the MIMS. Based on this understanding, a set of operational parameters designed to minimize the O_2 effect is recommended. This paper also seeks to address the following goals:

1. To determine the effect of O_2 concentrations on the calibration of the MIMS;

2. To develop a calibration appropriate for samples containing a wider range of O_2 concentrations;

3. To determine better experimental parameters that reduce the need for low O_2 corrections.

In addressing the first goal of this study, samples containing a range of O_2 concentrations will be analyzed for variations in NO^+ production as well as m/z 30 signal using MIMS. Elemental iron (Fe) will be used as a reductant and placed in a reactor with seawater in order to consume the ambient oxygen. A range of dissolved O_2 concentrations in each reactor will be achieved by using different temperatures and reaction times.

Data obtained during the first experiment will also be used to address the second goal of the paper, developing an appropriate calibration for $^{30}N_2$ determinations for samples containing a wider range of O_2 concentrations. Standardizing the $^{28}N_2$ signal to Ar, a physical tracer, is effective in maintaining the accuracy in N_2 measurements (Kana et al., 1994) and a similar method will be used to treat the $^{30}N_2$ calculations.

A brief survey of the MIMS literature was done in order to determine which parameters may influence the degree to which the O_2 effect is manifested in each instrument. An experiment was performed to verify the findings of the literature search.

3.3 Methods

3.3.1 Fe experiment

Seawater was collected in August 2005 from a shallow channel adjacent to the Skidaway Institute of Oceanography in Savannah, GA with a temperature of 27°C and a salinity of 19 per mil. The seawater was stored in a carboy in an environmental chamber set to 27°C. 20mL were pipetted into 24 batch tube reactors. Half of the reactors were placed in the 21°C water bath and the remaining reactors were placed in the 30°C water bath. These reactors remained open to the atmosphere and were allowed to equilibrate to ambient dissolved gas levels at the respective temperatures for 2 days.

Elemental iron (Fe) particles were sieved using a U.S.A. series #45 sieve. Approximately 0.5g of sieved Fe particles were placed in each of six of the reactors in the 21°C bath and six of the reactors in the 30°C bath. The remaining six reactors in each water bath were used as blank controls. All of the tubes were sealed with rubber stoppers and crimped shut with metal tops, creating gas-tight reactors. Each tube was shaken and placed horizontally, under water.

This experiment was performed two different times. During the first experiment, three reactors were sampled after 2 days and the remaining three were sampled after 3 days. Immediately upon opening, oxygen was measured using an O_2 mini-electrode (described below) and N_2 (including all of its isotopes) and Ar gases were measured

using MIMS. These measurements were done simultaneously (see MIMS and O_2 sections below). During the second experiment, all of the reactors were sampled in the same manner after 1 day. Prior to sampling, all of the reactors were shaken and allowed to stand upright in order for the Fe particles to settle to the bottom.

3.3.2 Membrane Inlet Mass Spectrometry (MIMS)

All measurements of N_2, including the isotopes of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$, and Ar were made on a membrane inlet mass spectrometer. The MIMS sampling system uses a peristaltic pump to direct water from the sample container to the membrane inlet. The sample water is routed through ~2 m of stainless steel (SS) capillary tubing that sits in a constant 21°C temperature bath. The SS tubing enters an evacuated housing and is connected to 3 cm of silastic tubing. The silastic tubing serves as a semipermeable membrane interface that allows dissolved gases to diffuse across the membrane and enter vacuum inlet system, after passing through a liquid N_2 cryotrap. The liquid N_2 trap is used to remove water vapor and CO_2, which can interfere with $^{28}N_2$ measurements through the production of various gas fragments in the mass spectrometer ion source (Kana, 1994). The gas sample then continues to the quadrupole mass spectrometer where masses 28, 29, 30, and 40 are detected (An, 2001). The output signal obtained from MIMS is given in units of ion currents.

3.3.3 Oxygen mini-electrode

Dissolved O_2 concentrations were determined using an MI-730 oxygen electrode attached to an OM-4 oxygen meter, both made by Microelectrodes, Inc. For the purpose of this

study, the electrodes were calibrated between 0 and 240 μM, the range of expected O_2 concentrations at 20°C. The 0 control was prepared by purging 20 mL seawater with N_2 gas for at least 30 minutes. Ambient seawater that was allowed to equilibrate with the atmosphere at 20°C for at least 24 hours was used for the 240 μM control.

The electrode was immediately inserted into each reactor after removing the top. In order to avoid atmospheric contamination of the sample, the electrode and MIMS capillary were simultaneously inserted near the bottom of each reactor.

3.3.4 Flow Rate Experiment

Instrument specific variations in the O_2 effect between different labs were hypothesized to be related to the number density of oxygen in the mass spectrometer. O_2 number density is, in turn, related to flow rates through the semi-permeable membrane in the MIMS. In order to provide further insight into the results of the Fe experiment, a second experiment was conducted in which the flow rate of fluid passing through the membrane was varied between 0.4 – 2.3 mL min^{-1}, to account for the variations found in the literature (Kana 1994, Nelson 2004, Bell 2007). The study by Bell et al. (2007) indicated a flow rate of 8 mL/min, which was beyond the capabilities of the peristaltic pump used in this study. Pressure, in units of torr, and the $^{28}N_2$ and O_2 signals were measured as a function of flow rate. The time that it takes for a sample to reach steady-state was also measured as a function of flow rate. An optimal range of flow rates was determined from the data.

3.3.5 NO^+ Formation

The total pressure in the ion source was measured on the MIMS during the flow rate experiment. This pressure was applied to the ideal gas law in order to calculate the number density for the total gas in the ion source:

$$PV = nRT \Rightarrow \frac{n}{V} = \frac{P}{RT} \qquad (3.2)$$

where n/V is concentration in mol L^{-1}, P is pressure in torr, R is the gas constant = 62.3637 in torr L K^{-1} mol^{-1}, T is the temperature in Kelvin.

The temperature in the ion source was assumed to be 292 K (Huey, personal communication), which was the room temperature in the laboratory that housed the MIMS.

The concentration of total gas, n/V, was converted into number density, with units of molecules cm^{-3}, using Avogadro's number. The calculated number density applied to the total gas in the system. The number densities of N_2 and O_2 were obtained by assuming that the gas sample was derived from air-saturated water containing 458 μmol/L N_2 and 249 μmol/L O_2 (Weiss 1970).

O^+ is formed in the ion course from the following reaction:

$$O_2 + e^- \rightarrow O^+ + O + 2e^- \qquad (3.3)$$

Electrons generated from the filament of the mass spectrometer used in this study obtain 22.5 eV of energy. The threshold energy for reaction 3.3 is 18.69 eV (Locht, 1974). The formation of O^+ ions within this MIMS system was thermodynamically possible.

NO^+ is produced within the ion source from the reaction between N_2 and O^+:

$$O^+ + N_2 \rightarrow N + NO^+ \qquad (3.4)$$

The production of NO^+ can be estimated using the second-order kinetics equation (Hanson, 1984):

$$\frac{d[NO^+]}{dt} = 2k[O^+][N_2] \qquad (3.5)$$

The rate constant, k, was determined to be 1.37 x 10^{-12} cm^3 (molecules*s)$^{-1}$, by extrapolating rate constants found for two different temperature ranges (Hierl, 1997; Le Garrec, 2003) to the temperature in which the reaction is occurring in this MIMS, 292 K. The number density of N_2 is determined from the measured pressure and the ideal gas law, as described above. For a given flow rate, the amount of O^+ produced is constant. Therefore, equation 3.5 can be integrated to give:

$$[NO^+] = k[N_2][O^+]\Delta t \qquad (3.6)$$

The ratio of $[NO^+]$ formed to a given amount of available $[O^+]$ can be estimated as:

$$\frac{[NO^+]}{[O^+]} = k[N_2]\Delta t \qquad (3.7)$$

The length of time of the reaction, Δt, can be estimated using the root mean square equation, applied to N_2 gas:

$$v_{rms} = \sqrt{\frac{3RT}{M}} \qquad (3.8)$$

where R can be converted to 1.38*10^{-23} J/K, M is the molecular mass for N_2, 28 amu x 1.67*10^{-27}kg/amu, and T is 292 K. The molecular speed of N_2 gas at 292 K is 508.5 m/s. The reaction time is simply found using the molecular speed and the distance that the N_2 molecules must travel in order to react with the ionized O atom, which is 1.5

cm for the MIMS used in this study. The reaction time is, t = d/v = 1.5 * 10^{-2} m/508.5 m/s = 2.94 * 10^{-5} s. Equation 3.7 can be simplified:

$$\frac{[NO^+]}{[O^+]} = 4.03 * 10^{-17} cm^3 molecule^{-1} [N_2] \qquad (3.9)$$

3.4 Results and Discussion

3.4.1 Anoxic Fe Experiment

The isotope tracer technique is based upon ^{15}N being amended to a system as $^{15}NO_3^-$ and/or $^{15}NH_4^+$ and being traced to the production of $^{29}N_2$ and/or $^{30}N_2$ as a result of various nitrification and denitrification pathways. This technique has been coupled with MIMS to become an effective method for measuring denitrification rates (An, 2001; Laursen, 2002; Vance-Harris, 2005; Rao, 2007).

The method of calculating $^{30}N_2$ concentrations from the MIMS output signal developed by An et al.(2001) considers the formation of NO^+ from N_2 and O_2 interactions within the ion source. The formation of NO^+ produces a m/z 30 signal, which can add to the m/z 30 signal from $^{30}N_2$, produced during isotope tracer experiments. Therefore, a relationship is established between the combined N_2 and O_2 signals and the m/z 30 signal for the standards. This relationship is represented by a linear correction equation, which is applied to the samples in order to avoid overestimating $^{30}N_2$ production due to NO^+ formation. The goal of this particular experiment is to determine whether corrections calculated with this approach are appropriate for low O_2 or anoxic systems.

In the present study, dissolved O_2 concentrations decreased significantly in the Fe reactors during both experiments. The production of NO^+, calculated by multiplying the

square root of the $^{28}N_2$ signal with the O_2 signal (An, 2001), decreases as O_2 concentrations decrease (Figure 3.1), as expected. Our results are consistent with those of Eyre et al. (2002) in that the observed relationship between NO^+ and O_2 is not linear. As O_2 concentrations approach 100 μM or less, the production of NO^+ decreases at an even more substantial rate (Figure 3.1). Calculated NO^+ production is used to determine $^{30}N_2$ concentrations. Therefore, this non-linearity would result in inaccurate calculations of $^{30}N_2$ concentrations.

The m/z 30 signal was plotted as a function of NO^+ production in our Fe reactors (Figure 3.2). The slope of this line is a key part in calculating $^{30}N_2$. The slope of the points is higher at lower O_2 concentrations (Figure 3.2). In typical experiments, calibrations are done with high O_2 solutions that would plot on the high end of figure 3.2. Extrapolation of this trend is clearly inappropriate for lower O_2 samples. Therefore, the standard method of calculating $^{30}N_2$ that relies on the production of NO^+ in the source is not suitable for samples containing 100 μM or less of dissolved O_2.

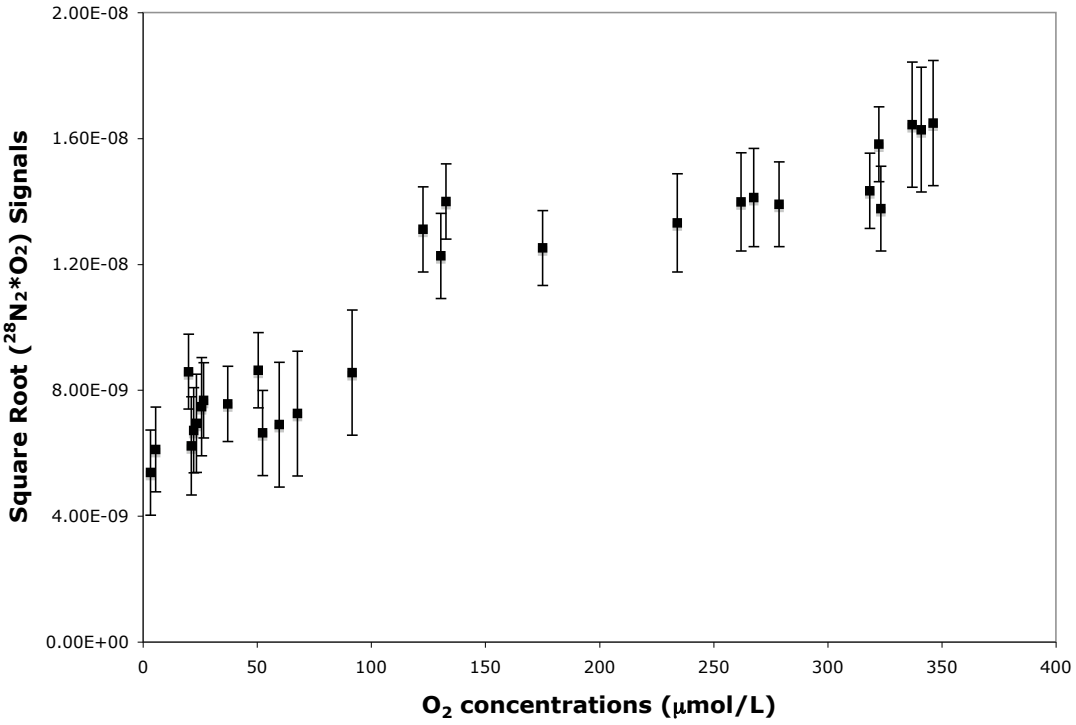

Figure 3.1: NO^+ vs. O_2 concentrations. The square root of $^{28}N_2$ * O_2 signals obtained from MIMS data is used as an approximation for NO^+ formation. NO^+ formation decreases as a function of O_2. However, the slope of NO^+ formation increases in standards containing O_2 concentrations between 0 and 100 μmol/L. These data, obtained from the Fe reactors, show the non-linearity of NO^+ formation with O_2 concentrations.

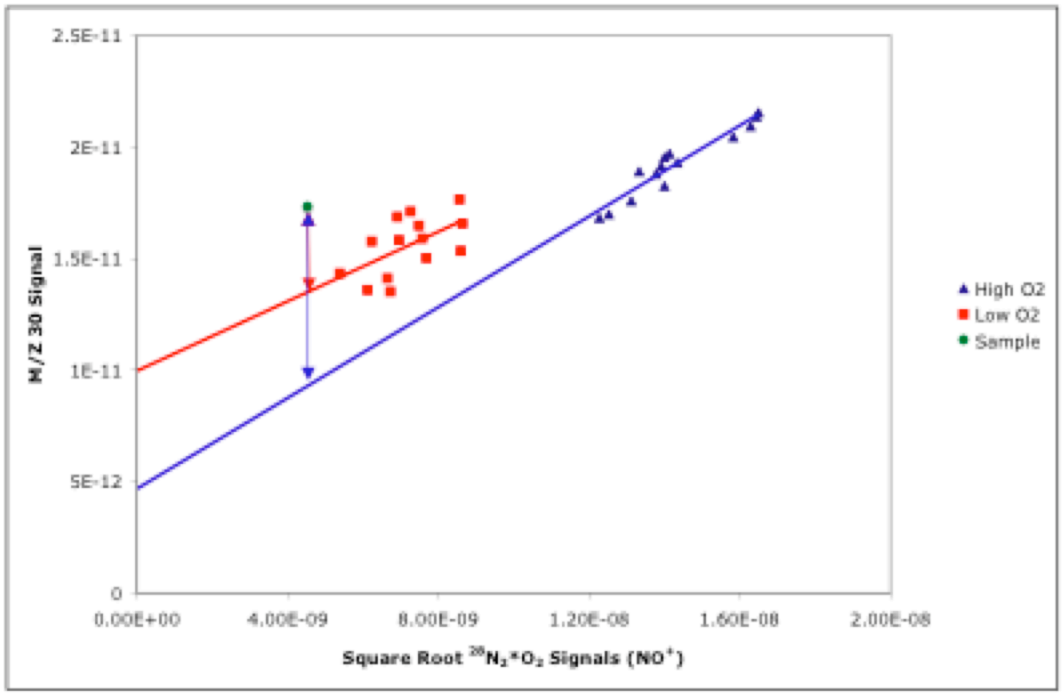

Figure 3.2: m/z 30 signal vs. NO^+ production. The accepted calculation method of $^{30}N_2$ concentrations measures the deviation of the m/z 30 signal of the sample (in green) from the trend extrapolated from high O_2 standards (in blue). Standards that contain lower O_2 concentrations (in red) produce higher m/z 30 signals. Overestimations of $^{30}N_2$ would result from using the method based on higher O_2 standards.

An improved method of calculating $^{30}N_2$ in environmental samples containing 100 μM or less of dissolved O_2 using MIMS is presented here and is based on the data obtained during the Fe experiment. The anoxic Fe samples were used as standards, as the concentrations of the other relevant gases (N_2 and Ar) are assumed to be unaffected by the addition of Fe to seawater. A relationship was established between the m/z 30:Ar and m/z 28:Ar signal ratios in the anoxic standards and an equation determined to estimate this relationship (equation 3.10). The data obtained during a denitrification experiment (unpublished data) was applied to this equation. An "excess m/z 30" signal was calculated by determining the difference between the measured m/z 30:Ar signal ratio in the sample and the estimated m/z 30:Ar signal ratio, which is established based

on the m/z 28 signal and the parameters established by the standards, slope and intercept (equation 3.11). The concentration of $^{30}N_2$:Ar is then estimated by multiplying the excess m/z 30 signal by the ratio of the expected $^{28}N_2$ concentration in the standards to the average $^{28}N_2$ signal of the standards of one temperature (equation 3.12), similar to the method used in the standard calculation of $^{30}N_2$ from MIMS data (An, 2001). The actual concentration of $^{30}N_2$ is calculated by multiplying the $^{30}N_2$:Ar concentration ratio by the expected Ar concentration at the same temperature (equation 3.13). We used data from the 30°C anoxic Fe samples, as this is closer to the original temperature of the samples, 27°C.

$$\frac{\left({}^{30}N_2\right)_{signal}}{\left(Ar\right)_{signal}} = \frac{\left({}^{28}N_2\right)_{signal}}{\left(Ar\right)_{signal}} * slope + \text{int}\, ercept \tag{3.10}$$

$$excess^{30}N_2 = \frac{\left({}^{30}N_2\right)_{signal}}{\left(Ar\right)_{signal}} - \left[\frac{\left({}^{28}N_2\right)_{signal}}{\left(Ar\right)_{signal}} * slope + \text{int}\, ercept\right] \tag{3.11}$$

$$\left[\frac{{}^{30}N_2}{Ar}\right] = excess^{30}N_2 * \frac{\left[{}^{28}N_2\right]_{\exp ected\,@\,21^\circ C}}{\left({}^{28}N_2\right)_{signal\,@\,21^\circ C}} \tag{3.12}$$

$$\left[{}^{30}N_2\right] = \left[\frac{{}^{30}N_2}{Ar}\right] * \left[Ar\right]_{\exp ected\,@\,21^\circ C} \tag{3.13}$$

The $^{30}N_2$:Ar method for calculating $^{30}N_2$ concentrations was used to correct values of $^{30}N_2$ produced during isotope tracer studies. The first study was the denitrification – nitrogen fixation experiment presented in Chapter 4. Figure 3.3a compares the values for $^{30}N_2$ calculated using the standard method (An, 2001) and the values obtained using the $^{30}N_2$:Ar method. The new values for $^{30}N_2$ are slightly lower than those obtained using the standard method. The second data set that was re-analyzed was from a study on

anammox and Mn-catalyzed nitrification (Newton, 2006), which reported relatively high rates of $^{30}N_2$ production. Figure 3.3b shows that the $^{30}N_2$:Ar method, again, results in lower $^{30}N_2$ concentrations.

These results show that the standard method of calculating $^{30}N_2$ may result in overestimations of denitrification. The calculation method presented here utilizes the physical tracer, Ar, as means of standardization. This method also allows for the analysis of samples containing a wider range of O_2 concentrations without altering the gas composition of the sample.

3.4.2 Flow Rate Experiment

The flow rate of the peristaltic pump used to inject liquid samples into the membrane inlet was adjusted to reflect the parameters used by several labs. The range of flow rates used was 0.4 – 2.3 mL/min. Pressure increased almost linearly with flow rate, with a steady-state pressure being reached at the higher flow rates (Figure 3.4).

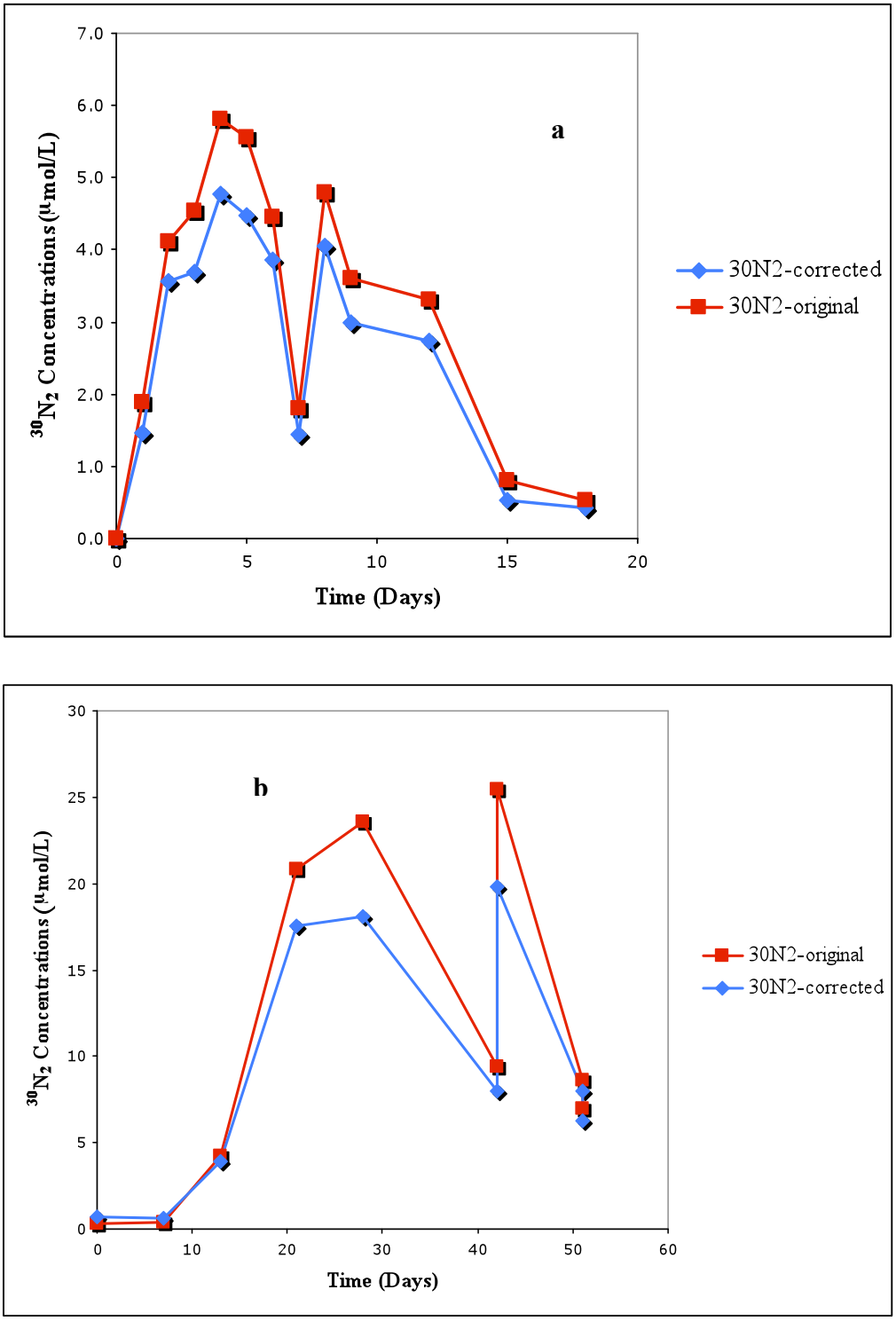

Figure 3.3: Standard method vs. $^{30}N_2$:Ar method for $^{30}N_2$ calculations. Data from both studies was analyzed using both methods. Standard method resulted in higher $^{30}N_2$ concentrations. a. Data presented in Chapter 4 from denitrification – nitrogen fixation experiment. B. Data from Newton (2006) during an anammox – Mn-catalyzed nitrification experiment.

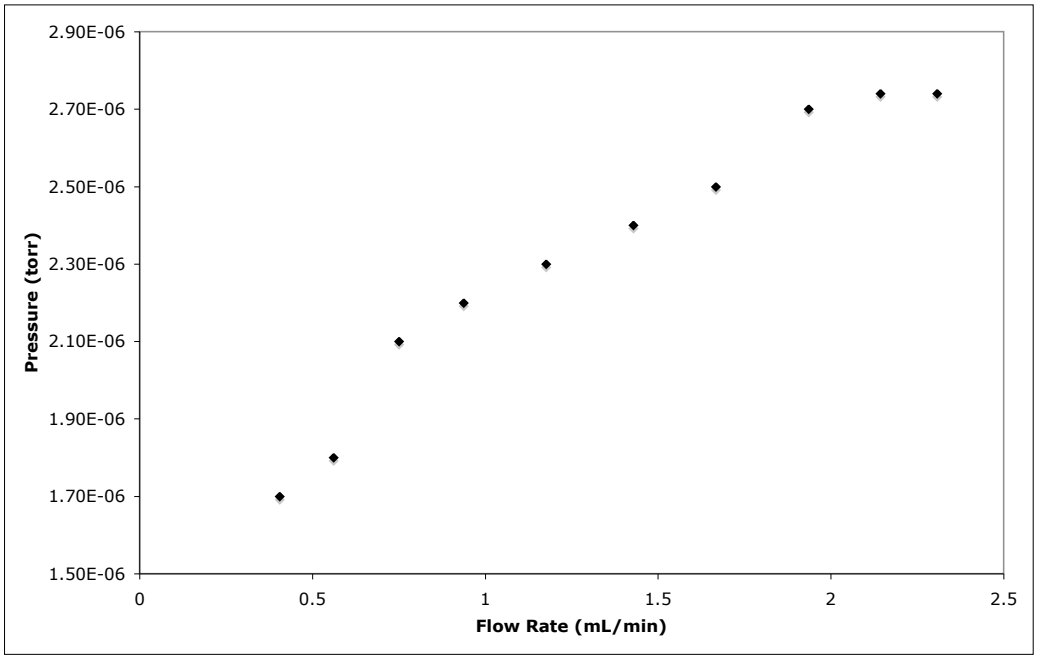

Figure 3.4: Flow Rate Experiment. Total pressure was measured on the MIMS as a function of the flow rate of fluid passing through the membrane inlet into the ion source.

3.4.3 NO^+ Formation

The pressures obtained during the flow rate experiment were used to calculate the number densities of N_2 (equation 3.2). Although the difference in measured pressures for the range of flow rates appeared to be small, the calculated range of N_2 number densities resulted in a 61% difference between the highest and lowest values (Table 3.1). These densities were then applied to equation 3.9.

Table 3.1: $[NO^+]/[O^+]$ Calculations. The pressure data obtained during the flow rate experiment is used to find N_2 number densities, using the ideal gas law, and applied to equation 3.9 to find $[NO^+]/[O^+]$ as a function of flow rate.

Flow Rate (mL/min)	P (torr)	n/V (molecules/cm^3)	n/V - N_2	$[NO^+]/[O^+]$
0	2.70E-07	8.89E+09	6.94E+09	2.80E-07
0.41	1.70E-06	5.60E+10	4.37E+10	1.76E-06
0.56	1.80E-06	5.93E+10	4.63E+10	1.86E-06
0.75	2.10E-06	6.92E+10	5.40E+10	2.18E-06
0.94	2.20E-06	7.25E+10	5.66E+10	2.28E-06
1.18	2.30E-06	7.58E+10	5.92E+10	2.38E-06
1.43	2.40E-06	7.91E+10	6.02E+13	2.42E-03
1.67	2.50E-06	8.23E+10	6.43E+10	2.59E-06
1.94	2.70E-06	8.89E+10	6.94E+10	2.80E-06
2.14	2.74E-06	9.03E+10	7.05E+10	2.84E-06
2.31	2.74E-06	9.03E+10	7.05E+10	2.84E-06

The quantity, $[NO^+]/[O^+]$, represents the amount of NO^+ formed from a given amount of O^+ produced in the mass spectrometer. The results show a 61% difference in $[NO^+]/[O^+]$ between the highest and lowest flow rates used (Figure 3.5). The highest flow rate used in this experiment, 2.31 mL/min, was much lower than the 8.0 mL/min flow rate used by Bell et al. (2007). The range of flow rates found in the literature is large enough to explain the differences in the O_2 effect experienced by different instruments.

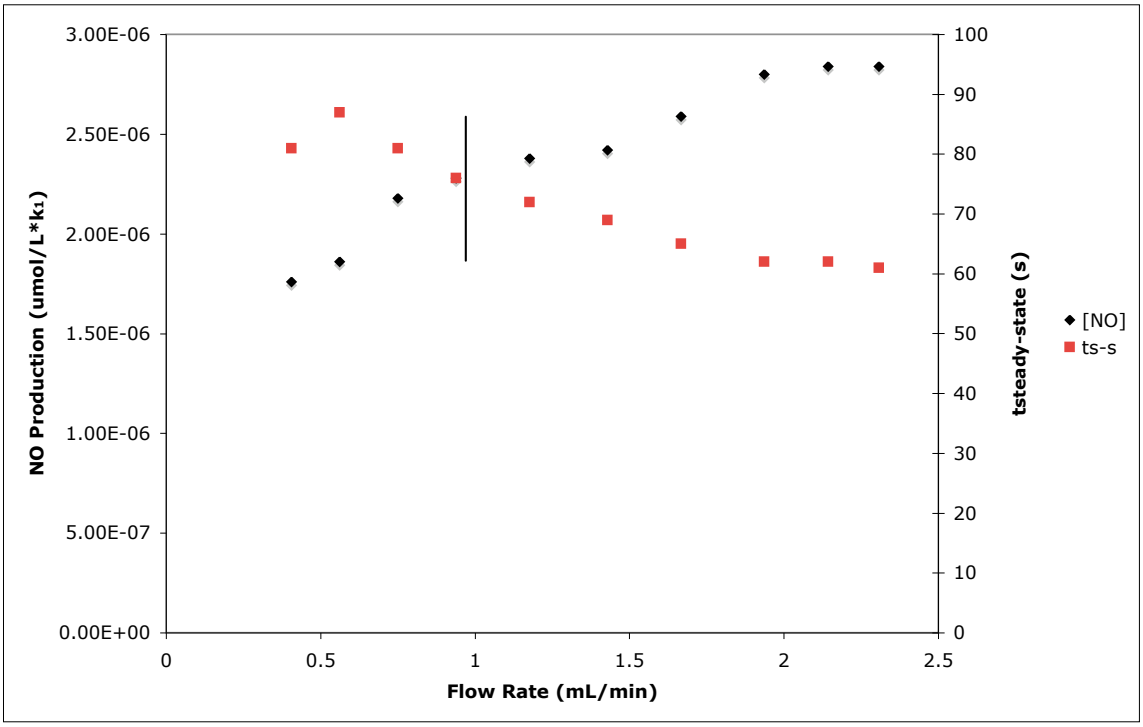

Figure 3.5: NO^+ production and Steady-State Time. The amount of NO^+ produced within the ion source was calculated from the pressure, which measured as a function of flow rate. The time that a sample takes to reach steady-state was also measured as a function of flow rate. Steady-state time and NO^+ production were used to determine an optimal flow rate of ~1 mL/min (indicated by the line).

Kana et al. (1994) suggest that higher flow rates result in less time for the signal to reach a steady-state. This parameter must be balanced with the higher NO^+ production also found using higher flow rates. The amount of time that the sample takes to reach steady-state was also evaluated as a function of flow rate. The time to steady-state generally decreased as flow rate increased (Figure 3.5). The greatest amount of time that a sample took to reach steady-state was 87 seconds, at a flow rate of 0.56 mL/min. Steady-state was reached in 61 seconds using a flow rate of 2.31 mL/min. A range of flow rates was determined in order to optimize the MIMS to produce lower NO^+ while analyzing samples in a timely fashion. A slower flow will decrease the O_2 effect, whilst an increased flow rate will minimize the time to reach steady-state. A flow rate of 1

mL/min produces only10% more NO^+ than that produced at 0.75 mL/min, the flow rate used by Kana et al. (1994) that did not show an O_2 effect. Steady-state was achieved in 76 s at 1 mL/min. The optimal flow rate for N_2 measurements using MIMS is 1 mL/min.

Another aspect of the MIMS that may contribute to the instrument-specificity of the O_2 effect is the actual physical construct of the MIMS. The length of the silastic tubing (silicone membrane) reported by different users varied between 4 and 5 cm (Brodbelt, 1987; Kana, 1994; Virkki, 1995). Kana et al. (1994) reported using silastic tubing with dimensions 0.3 mm ID (inner diameter) x 5 cm length. The volume of the tubing in their MIMS system is 14.1 mm^3. The dimensions of the silastic tubing for the MIMS used in the current study is 0.51 mm ID x 3 cm length, resulting in a volume of 24.5 mm^3, 74% greater than the volume for the Kana MIMS. The volume of the silastic membrane would regulate the amount of dissolved gas that diffuses into the ion source of the mass spectrometer and may, therefore, influence the response of the mass spectrometer to varying concentrations of dissolved gases. However, most of the published articles that incorporate MIMS analyses do not report such detail, making it difficult to assess the relevance of silastic tubing dimensions on the analytical capabilities of the instrument.

The instrument-specificity of the O_2 effect on N_2 measurements using MIMS appears to be the result of differences in NO^+ production as a function of flow rate. This finding is significant as it provides insight into the O_2 effect that has been reviewed in the literature without discussion on the mechanism responsible for the observations. Isotope-tracer and MIMS techniques are becoming more common for measurements of denitrification. These results will help to improve the overall understanding of

membrane inlet mass spectrometry, which will result in more accurate denitrification measurements.

3.5 Conclusions

Data collected using Fe to vary the concentration of dissolved O_2 in seawater determined that the MIMS used in this study displayed the O_2 effect, in which NO^+ is produced in the ion source as a result of interactions between N_2 and O_2. In order to calculate $^{30}N_2$, the production of NO^+ is accounted for and essentially removed from the m/z 30 signal. However, non-linearities in the relation between dissolved O_2 and NO^+ result in overestimations of $^{30}N_2$ at low O_2 concentrations. Therefore, the correction curve method of calculating $^{30}N_2$, which is measured during isotope tracer studies, is not valid. This study provides a method for calculating $^{30}N_2$ using anoxic Fe samples as standards and $^{30}N_2$/Ar ratios. This method was used to correct $^{30}N_2$ data generated from two isotope-tracer studies, which resulted in lower estimates of denitrification/anammox processes.

It has been suggested that the O_2 effect in MIMS measurements is instrument specific (Eyre, 2002; Kana, 2004; Rao, 2007). The study conducted by Kana et al. suggested that N_2:Ar can be accurately measured at various oxygen levels. In this study, we report that N_2:Ar is affected by O_2 concentrations. The instrument used in the Kana study reportedly maintained a relatively slow flow rate of liquid sample entering the membrane inlet. The MIMS used in this study maintained a slightly higher flow rate. Experimental data provided evidence that a flow rate of ~1mL/min is optimal for N_2 measurements using MIMS, balancing the O_2 effect and overall sampling time.

The goal of this study is to offer improvements for MIMS measurements of dissolved N_2 for a wider variety of samples. Improved measurements of N_2, including $^{30}N_2$ that is analyzed during isotope tracer studies, will help to better constrain denitrification measurements. Denitrification is the largest sink term for marine, fixed N (Devol, 1991; Middelburg, 1996; Pilson, 1998). Several studies suggest that the global, marine N budget is out of balance, with rates of losses exceeding rates of input (Codispoti, 1995; Middelburg, 1996; Codispoti, 2001). It is, therefore, imperative that the techniques used to measure denitrification are as ubiquitous and accurate as possible. It is our hope that the results of this study are used to further improve denitrification measurements on a global scale.

3.6 References

An, S., Gardner, W. S. and Kana, T. (2001) "Simultaneous Measurement of denitrification and nitrogen fixation using isotope pairing with membrane inlet mass spectrometry analysis." *Applied and Environmental Microbiology*. **67**(3), 1171-1178.

An, S., Gardner, W.S. and Kana, T. (2001) "Simultaneous Measurement of Denitrification and Nitrogen Fixation Using Isotope Pairing with Membrane Inlet Mass Spectrometry Analysis." *Applied and Environmental Microbiology*. **67**(3), 1171 - 1178.

Brodbelt, J. S., Cooks, R. G., Tou, J. C., Kallos, G. J., and Dryzga, M. D. (1987) "In Vivo Mass Spectrometric Determination of Organic Compounds in Blood with a Membrane Probe." *Analytical Chemistry*. **59**, 454-458.

Codispoti, L. A. (1995) "Is the ocean losing nitrate?" *Nature*. **376**, 724.

Codispoti, L. A., Brandes, J. A., Christensen, J. P., Devol, A. H., Naqvi, S. W. A., Paerl, H. W., and Yoshinari, T. (2001) "The oceanic fixed nitrogen and nitrous oxide budgets: Moving targets as we enter the anthropocene?" *Scientia Marina*. **65**, 85-105.

Devol, A. H. (1991) "Direct measurement of nitrogen gas fluxes from continental shelf sediments." *Nature*. **349**(6307), 319-321.

Eyre, B. D., Rysgaard, S., Dalsgaard, T. and Christensen, P. B. (2002) "Comparison of Isotope Pairing and N_2:Ar Methods for Measuring Sediment Denitrification - Assumptions, Modifications, and Implications." *Estuaries*. **25**(6A), 1077 - 1087.

Hanson, R. K. a. S., Siamak (1984). *Combustion Chemistry*. (J. Gardiner, William C.)**:** 361-421, Springer-Verlag.

Hierl, P. M., Dotan, I., Seeley, J. V., Van Doren, J. M., Morris, R. A., and Viggiano, A. A. (1997) "Rate constants for the reaction of O^+ with N_2 and O_2 as a function of temperature (300 - 1800 K)." *Journal of Chemistry and Physics*. **106**(9), 3540-3544.

Kana, T. M., and Weiss, D. L. (2004) "Comment on "Comparison of Isotope Pairing and N_2: Ar Methods for Measuring Sediment Denitrification: By B. D. Eyre, S. Rysgaard, T. Dalsgaard, and P. Bondo Christensen. 2002. *Estuaries* 25: 1077 - 1087." *Estuaries*. **27**, 173 - 176.

Kana, T. M., Darkangelo, C., Hunt, D. M., Oldham, J. B., Bennett, G. E. and Cornwell, J. C. (1994) "Membrane Inlet Mass Spectrometer for Rapid High-Precision Determination of N_2, O_2, and Ar in Environmental Water Samples." *Analytical Chemistry*. **66**, 4166 - 4170.

Kana, T. M., Darkangelo, C., Hunt, M. D., Oldham, J. B., Bennett, G. E. and Cornwell, J. C. (1994) "Membrane inlet mass spectrometry for rapid high-precision determination of N_2, O_2, and Ar in environmental water samples." *Analytical Chemistry*. **66**(23), 4166-4170.

Laursen, A. E., and Seitzinger, S. P. (2002) "The role of denitrification in nitrogen removal and carbon mineralization in Mid-Atlantic Bight sediments." *Continental Shelf Research*. **22**, 1397 - 1416.

Le Garrec, J.-L., Carles, S., Speck, T., Mitchell, J. B. A., Rowe, B. R., amd Ferguson, E. E. (2003) "The ion-molecule reaction of O^+ with N_2 measured down to 23 K." *Chemical Physics Letters*. **32**, 485-488.

Locht, R., and Schopman, J. (1974) "The Dissociative Ionization in Oxygen." *International Journal of Mass Spectrometry and Ion Physics*. **15**, 361-378.

Middelburg, J. J., Soetaert, K., Herman, P. M. J. and Heip, C. H. R. (1996) "Denitrification in marine sediments: A model study." *Global Biogeochemical Cycles*. **10**(4), 661-673.

Newton, J. D. (2006). Evidence for manganese-catalyzed nitrogen cycling in salt marsh sediments. School of Earth and Atmospheric Sciences. Atlanta, GA, Georgia Institute of Technology. **Master of Science:** 80.

Pilson, M. E. Q. (1998). *An Introduction to the Chemistry of the Sea*. Prentice-Hall, Inc. 431.

Rao, A. M. F., McCarthy, M. J., Gardner, W. S. and Jahnke, R. A. (2007) "Respiration and denitrification in permeable continental shelf deposits on the South Atlantic Bight: Rates of carbon and nitrogen cycling from sediment column experiments." *Continental Shelf Research*. **27**, 1801 - 1819.

Thomas, C. R., and Christian, R.R. (2001) "Comparison of nitrogen cycling in salt marsh zones related to sea-level rise." *Marine Ecology-Progress Series*. **221**, 1-16.

Tortell, P. D. (2005) "Dissolved gas measurements in oceanic waters made by membrane inlet mass spectrometry." *Limnology and Oceanography: Methods*. **3**, 24 - 37.

Vance-Harris, C., and Ingall, E. (2005) "Denitrification pathways and rates in the sandy sediments of the Georgia continental shelf, USA." *Geochemical Transactions*. **6**(1), 12 - 18.

Virkki, V. T., Ketola, R. A., Ojala, M., Kotiaho, T., Komppa, V., Grove, A., and Facchetti, S. (1995) "On-Site Environmental Analysis by Membrane Inlet Mass Spectrometry." *Analytical Chemistry*. **67**, 1421-1425.

CHAPTER 4

SEASONAL VARIATIONS OF NITROGEN CYCLING MECHANISMS IN GEORGIA (USA) SALT MARSH SEDIMENTS

4.1 Abstract

Heterotrophic denitrification and nitrogen fixation were measured concurrently in the salt marsh sediments of Skidaway Island, Georgia, USA. Sediments from a mud flat and water from an adjacent channel were collected in the summer and winter. Isotope tracer experiments were carried out on sediments containing the roots and rhizomes of *Spartina alterniflora*. $^{15}N\text{-}NO_3^-$ and $^{15}N\text{-}NH_4^+$ were used to amend the system in order to measure isotopically distinct end products, $^{29}N_2$ and $^{30}N_2$, which help to distinguish between different N removal pathways. Denitrification and N_2 fixation were simultaneously measured using membrane inlet mass spectrometry (MIMS). Rates of both processes were relatively low in the winter in comparison to rates obtained during the summer. MIMS data indicated that N_2 fixation returns ~90% of the N_2 generated during denitrification back into the fixed N pool during the winter. This data suggests that these processes are occurring at relatively the same rate and that the net amount of fixed N remains unchanged in Georgia salt marsh sediments in the winter. Heterotrophic N_2 fixation by sulfate-reducing bacteria appears to be the mechanism of biological N_2 fixation in these sediments. Seasonal variations in the processes controlling N cycling in these sediments were also observed. Heterotrophic denitrification was found to be the dominant pathway of fixed N removal during both seasons. However, anaerobic ammonium oxidation was measured during the summer but was not observed in the

winter. These findings suggest that N exhibits seasonal variations with respect to the processes within the N cycle as well as the rates at which the processes occur.

4.2 Introduction

Salt marshes are quite ubiquitous along the eastern coast of the United States and serve as habitats for fisheries and many marine organisms. With respect to marine organisms, these environments are very productive and are thought to be limited by amounts of biologically available, or fixed, nitrogen, N. Denitrification, the reduction of nitrate, NO_3^-, and nitrite, NO_2^- (equation 4.1), limits productivity within the marsh, as most organisms are not able to assimilate nitrogen gas for growth (Valiela, 1979; Joye, 1994; Currin, 1995; Currin, 1996; Thomas, 2001; Eriksson, 2003; Hammersley, 2005; Wang, 2007). Nitrogen fixation is the process whereby dinitrogen, N_2, gas is taken up by an organism and converted to more bioavailable species of N such as nitrate and ammonia and has also been widely regarded as an important process in salt marshes (Jones, 1974; Herbert, 1975; Carpenter, 1978; Teal, 1979; Valiela, 1979; Nedwell, 1980; Whiting, 1986; Gandy, 1988; O'Donohue, 1991; Newell, 1992; Joye, 1994; Currin, 1996; Herbert, 1999; Nielsen, 2001; Thomas, 2001; Marino, 2002; Steppe, 2002; Tyler, 2003). The balance of denitrification and nitrogen fixation controls levels of bioavailable N and, therefore, overall productivity in salt marshes, as well as import and export of N into and out of the system. These two opposing processes may occur concurrently within the ecosystem of the marsh.

$$5CH_2O + 4NO_3^- + 4H^+ \rightarrow 2N_2 + 5CO_2 + 7H_2O \qquad (4.1)$$

In response to high [NO_3^-] from terrestrial inputs, denitrification in estuarine systems has been shown to effectively remove as much as 75% of this excess N from entering coastal waters (Scott, 2008). The removal of NO_3^- via denitrification may enhance estuarine N_2 fixation, leading to a return in excess N in the form of organic N, which may be transported to the coastal watershed. Although high N loads enter some estuarine systems, the co-existence of denitrifying and N_2-fixing bacteria that have been found in these sediments and rhizomes may be able to balance one another, preventing net fluxes of fixed N into or out of these environments (Valiela, 1979).

The exportation of fixed N from the salt marsh to the coastal ocean, implying that the marsh is a source of fixed N, would result in enhanced oceanic primary production. Studies conducted on Sapelo Island, Georgia, salt marshes, however, indicate that the marsh is a net sink of N (Cai, 2000; Thomas and Christian, 2001). Thomas and Christian present data suggesting that the NO_3^- imported into the Sapelo Island low marsh is completely removed via denitrification. Primary production was largely controlled by the input from precipitation in the low marsh of Sapelo Island. Another study conducted by Brion et al. (2008) indicated that 50 – 60 % of the N imported into a Dutch estuary is removed by anaerobic respiration (denitrification) with a relatively large portion (30 – 40%) being exported to the North Sea during most of the year. However, during the spring, an increased biomass density results in a large portion of fixed N being consumed by the microorganisms in the estuary. The estuary, in this scenario, is an efficient recycler of fixed N. Gaining insight into whether the marsh serves as a net source or sink of fixed N is important for determining the factors that control productivity for that area.

The biogeochemistry of the salt marsh/estuarine system of Georgia has been extensively studied (Cai, 2000; Castro, 2003; Korestsky, 2003; Kotska, 2002; Lowe, 2000; Newton, 2006; Roychoudry, 1999). Anaerobic metabolism is the primary means of organic matter mineralization because of the high organic matter content of these salt marsh sediments (3 – 4 % by weight, Roychoudry, 1999), which causes rapid utilization of dissolved O_2 in the top few mm (Luther, 1996). Sulfate reduction is thought to be the dominant pathway of anaerobic respiration in salt marsh sediments (Howarth, 1993). Seasonal variations in respiration rates and pathways, specifically iron and sulfate reduction, were observed in salt marsh sediments at Sapelo Island, Georgia, USA (Korestky, 2003; Kotska, 2002). These seasonal variations may influence N cycling in two ways:

Sulfate reducing bacteria (SRB) have been shown to be the dominant N_2-fixers in salt marsh sediments (Jones, 1974; Herbert, 1975; Teal, 1979; Valiela, 1979; Nedwell, 1980; Gandy, 1988; O'Donohue, 1991; Nielsen, 2001; Steppe, 2002). Decreased rates of sulfate reduction during the winter were measured in Georgia salt marsh sediments in comparison to rates measured during the summer (Kostka, 2002; Koretsky, 2003). The seasonal variation in SRB activity may cause corresponding fluctuations in N_2 fixation rates.

Secondly, fixed N removal can occur through a number of pathways. One pathway of fixed N removal is heterotrophic denitrification, which is an anaerobic respiration process. Denitrification rates may therefore reflect similar seasonal patterns of overall respiration rates.

N cycling studies in Georgia salt marshes have revealed that fixed N removal

pathways other than canonical heterotrophic denitrification are relevant in this ecosystem (Cai, 2000; Newton, 2006). The study by Cai (2000) determined that nitrification, the aerobic oxidation of ammonium to nitrate, may be responsible for the high NO_3^- concentrations observed in the Satilla River marsh system. A portion of the NO_3^- derived from nitrification is subsequently denitrified, which is known as coupled nitrification-denitrification (Jenkins, 1984). Newton (2006) discovered that manganese (Mn)-catalyzed nitrification (equations 4.2 a, b) coupled to anammox (anaerobic ammonium oxidation, equation 4.3), which results in N_2 production, is responsible for fixed N removal in Skidaway Island salt marsh sediments. These two studies were conducted during the summer and autumn, therefore not considering any seasonal fluctuations in the biogeochemistry of the salt marsh that is certain to influence the pathways and rates of the local N cycle.

$$4MnO_2 + NH_4^+ + 6H^+ \rightarrow 4Mn^{2+} + NO_3^- + 5H_2O \qquad (4.2\ a)$$

$$3MnO_2 + 2NH_4^+ + 4H^+ \rightarrow 3Mn^{2+} + N_2 + 6H_2O \qquad (4.2\ b)$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad (4.3)$$

It is not clear what effect concurrent denitrification and N_2 fixation processes have on local N budgets. In this study, N cycling pathways were examined in the Skidaway Island salt marsh in Savannah, Georgia. Experiments were conducted during the summer and winter in order to determine any seasonal fluctuations of N cycling that may result from the anaerobic respiration variability, specifically the mechanism of fixed N removal and the rates of both denitrification and N_2 fixation.

The unvegetated mud flat of Skidaway Island, Savannah, Georgia is most similar to the low marsh at Sapelo Island in Georgia, where a number of studies on anaerobic

respiration were conducted (Taillefert, 2007; Thomas and Christian, 2001; Kostka, 2002; Korestky, 2003). The low marsh exhibited average rates for most N processes as well as total system throughput as compared to the creek bank and high marsh. In order to facilitate a meaningful discussion regarding N cycling as it relates to the trends observed at Sapelo Island, samples were obtained from the unvegetated mud flat at Skidaway Island and isotope tracer experiments were conducted on these sediments. Isotope tracer experiments were performed in order to distinguish between the pathway of fixed N removal, which determines the production of isotopically distinct end products, $^{29}N_2$ and $^{30}N_2$. Dissolved gas analyses of N_2 and O_2 were done using membrane inlet mass spectrometry (MIMS). Denitrification and nitrogen fixation were estimated using MIMS data, according to An et al. (2001) and described in 4.3.5.

4.3 Methods

4.3.1 Sampling

The top 4 cm of sediment was collected from an unvegetated mud flat in a salt marsh on Skidaway Island in Savannah, Georgia in August 2005 and February 2008 (Figure 4.1). The salt marsh is located in the Salt marsh Ecosystem Research Field (SERF) station. This particular location represents a pristine salt marsh, relative to other salt marshes along the U.S. east coast. The mud flat is located only meters away from a portion of the marsh vegetated by *Spartina alterniflora*. Characteristics of the salt marsh sediment can be found in Taillefert et al. (2007).

Figure 4.1: Skidaway Island salt marsh (reprinted from Taillefert et al. 2007). Samples were collected from the mud flat (MF) during low tide in August 2005 and February 2008.

Sampling was done during low tide. The temperature and salinity of the sediment and overlying water were 27°C and 35 psu in August and 19°C and 28 psu in February. Seawater from the adjacent channel was also collected. The sediment and seawater samples were driven back to the laboratory at Georgia Tech in Atlanta, GA and stored in an environmental chamber at ambient temperature.

4.3.2 Incubation Experiment

In order to determine the occurrence of N_2 fixation and identify the pathway of fixed N removal in Georgia salt marsh sediments, incubation experiments were set up in anaerobic batch reactors. Sediment was homogenized by manually stirring the sediment with a spatula. Roughly 5mL of homogenized sediment was weighed and then added to each reactor. 17 mL of different seawater solutions was added to each tube containing sediment. Each reactor was shaken after being sealed and remained static until sampled.

4.3.2.1 Summer Incubation

For the August incubation experiment, a 50 μM solution of $^{15}N\text{-}NH_4^+$ was used to amend the system. A control set was established by incubating the sediment in unamended seawater. Duplicate reactors were set up for each treatment and sampling point. Sampling took place daily during the first 7 days, then every 3 days for the next 15 days, then every 7 –10 days for the remainder of the incubation.

4.3.2.2 Winter Incubation

For the February incubation, 100 μM solutions of $^{15}N\text{-}NO_3^-$ and $^{15}N\text{-}NH_4^+$ were used to distinguish the pathway of N_2 production. Canonical heterotrophic denitrification utilizes NO_3^- as a substrate and would produce quanitifiable amounts of $^{30}N_2$ using the $^{15}N\text{-}NO_3^-$ solution. Anammox utilizes NH_4^+ as a substrate to produce $^{29}N_2$ from the reaction between $^{15}N\text{-}NH_4^+$ and ambient NO_2^- using the $^{15}N\text{-}NH_4^+$ solution. NH_4^+ can also be oxidized to NO_2^- and NO_3^- by Mn-oxides (Luther, 1997; Hulth, 1999), which would then undergo heterotrophic denitrification and/or anammox (Newton, 2006). $^{29}N_2$ and $^{30}N_2$

would be produced as a result of this pathway using the $^{15}N\text{-}NH_4^+$ solution. Reactors were sampled daily during the first 9 days and then every 3 days during the remaining 9 days.

4.3.3 Nutrient Analyses

Dissolved NH_4^+, NO_2^-, and NO_3^- were determined colorimetrically and analyzed using a UV/Vis spectrophotometer (Hansen, 1999). Hydrogen sulfide (H_2S) was determined spectrophotometrically as methylene blue (Fonselius, 1999).

4.3.4 Dissolved Gas Analyses

Membrane inlet mass spectrometry was used to measure $^{28,29,30}N_2$, O_2, and Ar. Details of the instrumentation and technique can be found in Chapter 3 and Vance-Harris and Ingall (2005).

4.3.5 Denitrification and N_2 Fixation Rates

Rates of heterotrophic denitrification and N_2 fixation were calculated using the method developed by An et al. (2001) in which the two processes are measured simultaneously using isotope tracer and MIMS techniques. Briefly, concentrations of $^{29}N_2$ and $^{30}N_2$ are used to determine the rate of production over time. The An et al. method was originally applied to flow through sediment incubation experiments and the flow rate of inflow solution was used to calculate the production rate of N_2. In this study, static, batch reactors were used and the method was, therefore, altered to accommodate the static incubation experimental design.

Trimmer et al. (2003) measured anammox with static batch reactors and the isotope tracer technique. Rates of $^{29}N_2$ and $^{30}N_2$ were determined in units of nmol N_2 mL^{-1} wet sediment hr^{-1} using a simple equation of N_2 production per unit time (equation 4.1). Simple unit conversions were done in order to compare measured rates with those obtained by Newton (2006) where anammox was recently measured within meters of the current study.

$$\frac{d[N_2]}{dt} = \frac{[^{29}N_2] + [^{30}N_2]}{t} \qquad (4.1)$$

The $^{29,30}N_2$ production rates were used to determine total denitrification rates and N_2 fixation rates (An, 2001).

4.4 Results

4.4.1 Incubation Experiments

4.4.1.1 Summer Incubation

The addition of $^{15}NH_4^+$ tracer resulted in the formation of $^{29}N_2$ and $^{30}N_2$, after 15 days of incubation (Figure 4.2 a). Significant decreases in N_2 were also observed, immediately following production. The production of N_2 was concurrent with a decrease in $[NH_4^+]$ and an increase in $[NO_3^-]$ (Figure 4.2 b).

4.4.1.1 Winter Incubation

Sediment incubations amended with the $^{15}N\text{-}NO_3^-$ solution lead to production of $^{29}N_2$ and $^{30}N_2$ after 24 hours (Figure 4.3 a). $^{30}N_2$ continued to increase over the next 7 days.

Nutrient analyses indicated a decrease in NO_2^- and NO_3^- from the initial concentration to zero within the first 48 hours. NH_4^+ concentrations increased steadily over the initial 7-day period (Figure 4.3 b). MIMS data showed a significant decrease in both $^{29}N_2$ and $^{30}N_2$ on day 7, based on duplicate anaerobic batch reactors. The sudden decrease in N_2 was accompanied by a substantial increase in NH_4^+. $^{29}N_2$ and $^{30}N_2$ continued to decrease during the latter days of the incubation (Figure 4.3 a). The decrease in N_2 was accompanied by an increase in NH_4^+ concentrations (Figure 4.3 b).

H_2S was produced and measured in all three treatments. The results from the ^{15}N-NO_3^- treatment are shown in Table 4.1. H_2S appears on day 5 and is at a maximum concentration of 18 μM on day 15.

Dissolved O_2 was measured on the MIMS for all days during the entire length of the experiment. However, H_2S was measured on or after day 5 for all three treatments. The presence of H_2S nullifies the dissolved O_2 data, as H_2S is a product of sulfate reduction, an anaerobic respiration pathway. It can, therefore, be assumed that the dissolved O_2 concentrations reach zero by, at least, day 5 in the ^{15}N-NO_3^- treatment and remain anoxic throughout the remainder of the incubation. The O_2 data in Figure 4.3a has been corrected to reflect the assumed anoxic conditions.

Incubations amended with the ^{15}N-NH_4^+ solution showed no measurable production of either ^{29}N or ^{30}N-N_2. The dissolved inorganic N data was similar to that measured for the ^{15}N-NO_3^- solution (Data not shown).

4.4.2 Denitrification and N_2 Fixation Rates

An average denitrification rate was calculated to be 0.031 nmol N_2 mL^{-1} wet sed. hr^{-1} for

the entire 18 days of the incubation experiment. An average N_2 fixation rate was calculated only for those days when decreases in $^{29}N_2$ and $^{20}N_2$ were measured. The average N_2 fixation rate was 0.040 nmol N_2 mL^{-1} wet sed. hr^{-1}.

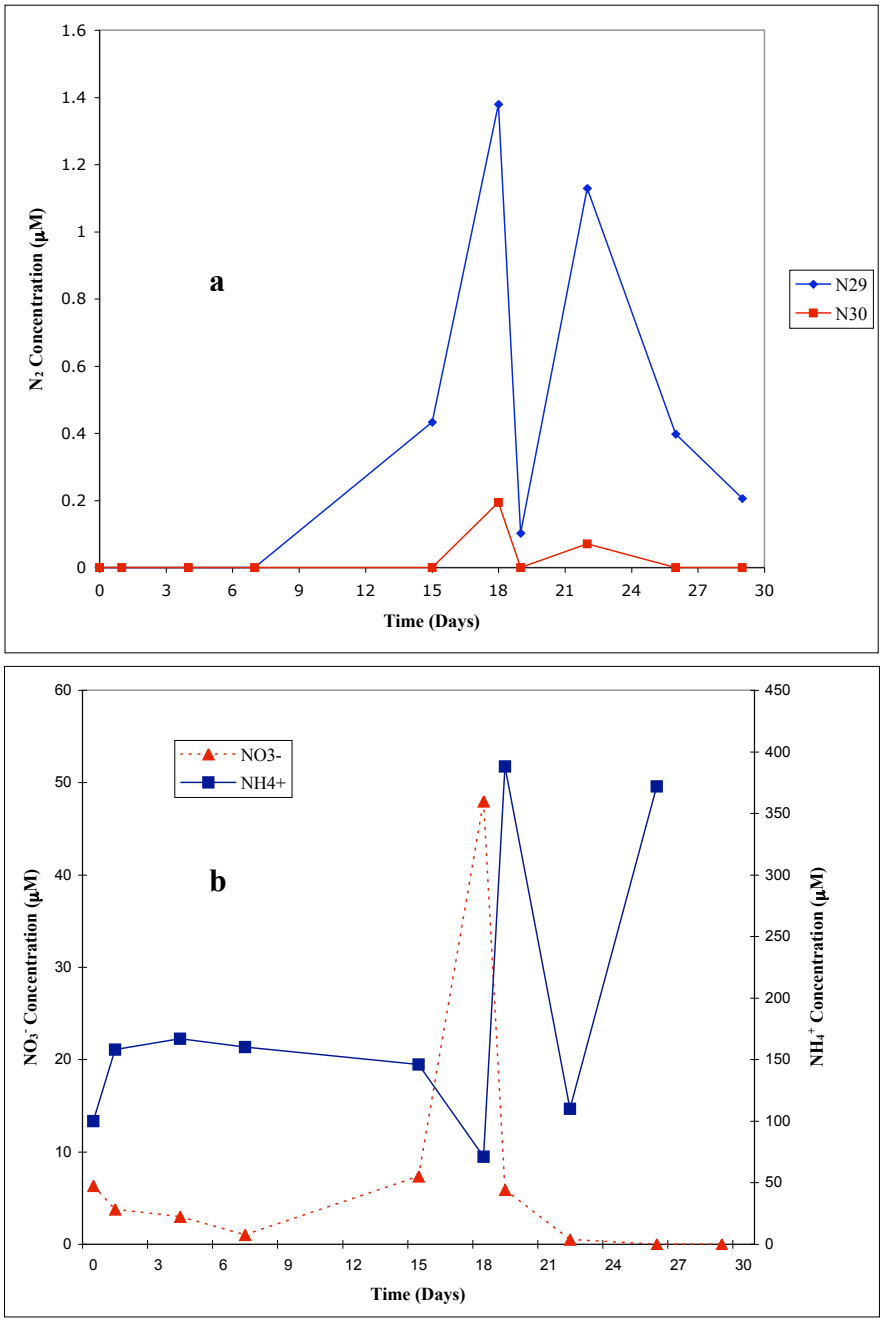

Figure 4.2: Summer Incubation. a. Dissolved gas data. $^{15}NH_4^+$ amended incubations led to production of $^{29}N_2$, followed by a substantial decrease in N_2.
b. Dissolved inorganic N data. $[NH_4^+]$ decreases as $[NO_3^-]$ increases. These data indicate the presence of anaerobic ammonium oxidation.

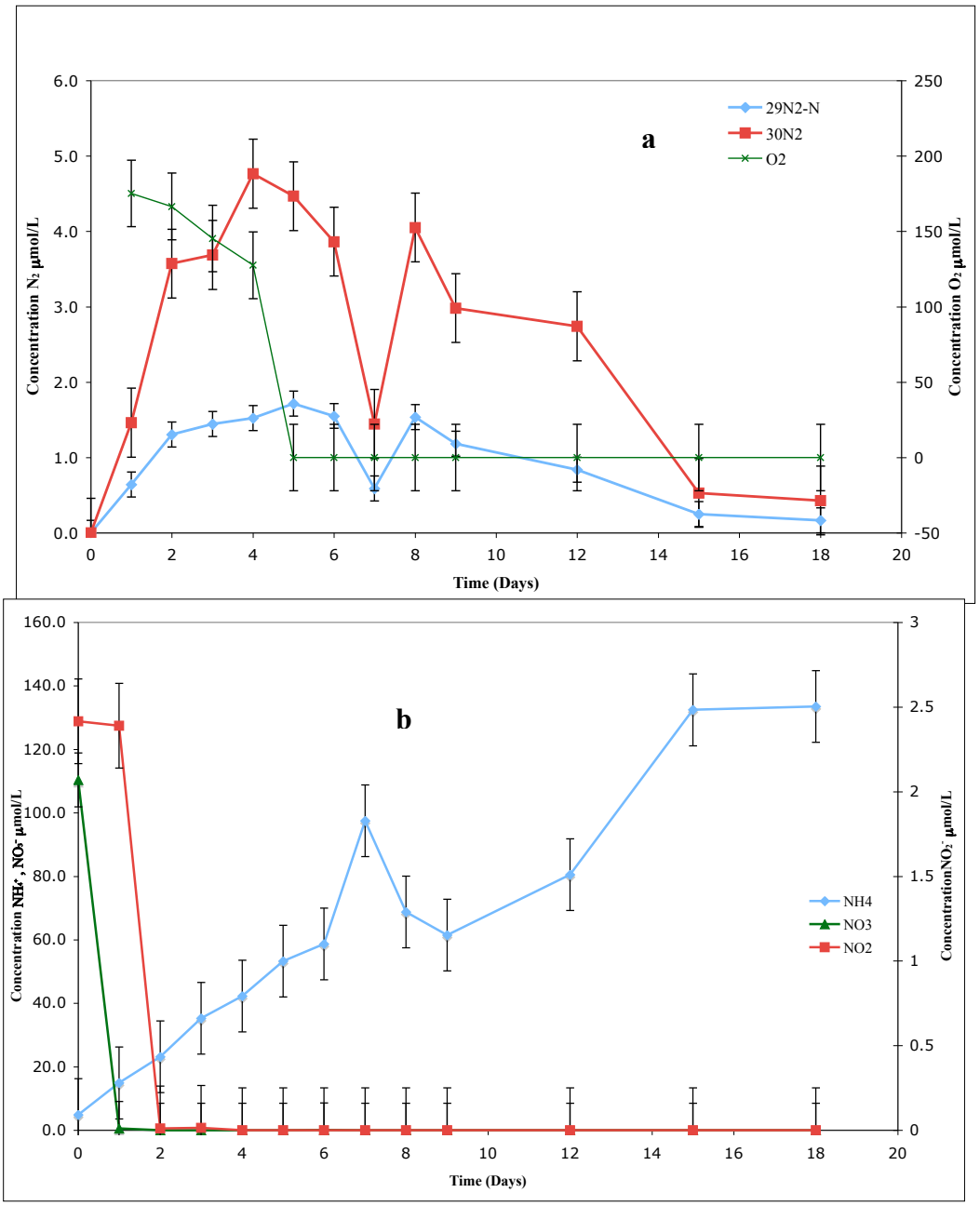

Figure 4.3: Winter Incubation. a. Dissolved Gas Data. Membrane inlet mass spectrometry (MIMS) was used to determine concentrations of dissolved $^{29}N_2$, $^{30}N_2$, and O_2. $^{29,30}N_2$ produced as a result of isotope tracer experiments reflect an initial period dominated by heterotrophic denitrification, followed by a decrease in N_2, indicative of N_2 fixation.
b. Dissolved inorganic N data. NO_2^- and NO_3^- are consumed within the first two days of the incubation, indicating the presence of denitrification. A sharp increase in NH_4^+ is concurrent with decreases in $^{29,30}N_2$. All measurements are based on duplicate reactors.

Table 4.1: Hydrogen Sulfide in the $^{15}N\text{-}NO_3^-$ treatment (average of duplicate reactors). Production of H_2S indicates that dissolved O_2 is most likely not present.

Day	H_2S (μmol/L)
0	0
4	0.277294151
5	1.510616043
7	3.596889524
15	18.0279083
18	10.2245539

4.5 Discussion

4.5.1 Nitrogen removal pathway

The production of $^{29}N_2$ and $^{30}N_2$ from sediment incubations during the winter containing the solution amended with $^{15}N\text{-}NO_3^-$ indicate that canonical heterotrophic denitrification exists in these salt marsh sediments. The results from incubations using the solution amended with $^{15}N\text{-}NH_4^+$ indicate that NH_4^+ oxidation leading to N_2 production through Mn-catalyzed nitrification and anammox pathways was not occurring in these sediments during the winter. However, summer data suggested the existence of some pathway involving NH_4^+ oxidation leading to N_2 production (Figure 4.2 a,b). Also, a study conducted by Newton (2006) suggests that both anammox and Mn-catalyzed nitrification do occur within these same sediments, collected within meters of the sampling site of this study. These previous incubations were conducted during the summer. Both studies utilized isotope tracer and MIMS techniques. Seasonal variations in anaerobic metabolism may explain these observations. Previous studies show much lower bacterial N_2 fixation rates in salt marsh sediments during the winter, with no measurable rates in some cases (Jones, 1974; Herbert, 1975).

Anammox seems to occur and persists in sediments containing high

concentrations of NO_2^- and/or NO_3^- (Risgaard-Petersen, 2005). During the winter incubation, the amended $^{15}NO_3^-$ was utilized within the first day of the incubation through heterotrophic denitrification, preventing the persistence of high NO_3^- concentration necessary for anammox to take place. During the experiments conducted by Newton (2006), high concentrations of NO_x persisted for more than 50 days, most likely evolved during Mn-catalyzed nitrification, thus allowing favorable conditions for anammox to occur.

The potential for heterotrophic denitrification, anammox, Mn-catalyzed nitrification, and heterotrophic N_2 fixation exists in the sediments of the Skidaway Island salt marsh. The bacteria that perform these functions are all present within this ecosystem. It is not clear, however, that they are all active at the same time. During the Newton summer study, it appeared that high Mn-oxide levels (amended to the sediments) were responsible for the occurrence of the Mn-catalyzed nitrification, which produced high levels of NO_x. Anammox appeared to result from the high NO_x levels that persisted during the 50+ day incubation.

4.5.2 Heterotrophic N_2 Fixation

N_2 fixation has previously been detected and measured in Georgia salt marshes (Newell, 1992; Lovell, 2001). These measurements were made on pieces of standing, dead *Spartina* shoots during the spring, summer, and autumn months. The study conducted by Newell et al. (1992) determined that heterotrophic bacteria may be responsible for the observed activity of nitrogenase, the enzyme found in all nitrogen-fixing organisms that catalyzes the breakdown of dinitrogen molecules. During the current winter study,

organic matter was oxidized via heterotrophic denitrification. Electrons are released as the result of oxidation reactions, which may have activated the heterotrophic N_2 fixers (Nedwell, 1980). Similar N_2 trends, showing substantial decreases after several incubation days, were observed during the summer and winter, as well as by Newton (2006) (Figure 3.3 b). Organic matter is readily available for oxidation reactions within these sediments. High concentrations of potential electron acceptors such as NO_3^- (Figures 4.2b, 4.3b) and SO_4^{2-} (Data not shown) were available during both experiments. The availability of electrons produced during organic matter oxidation allows heterotrophic N_2 fixation to occur at all times of the year. The production of H_2S (Table 4.1) provides evidence for the activity of the sulfate-reducers, which are the dominant N_2-fixers in salt marsh sediments (Jones, 1974; Herbert, 1975; Teal, 1979; Valiela, 1979; Nedwell, 1980; Gandy, 1988; O'Donohue, 1991; Nielsen, 2001; Steppe, 2002). These data suggest that heterotrophic N_2 fixation by sulfate-reducers contributes to new fixed N in Georgia salt marsh sediments.

4.5.3 Seasonal Variations

Denitrification rates obtained during the winter in the current study are an order of magnitude lower than those obtained during the summer, as reported by Newton (2006) (0.031 vs. 0.87 nmol N_2 mL^{-1} wet sed. hr^{-1}, respectively). The lower denitrification rates found in winter are consistent with the seasonal trends in anaerobic respiration rates measured by Kostka (2002) in the salt marsh of Sapelo Island, Georgia.

N_2 fixation has been studied in a variety of salt marsh and estuarine environments. Salt marsh fixation has been reported to occur via two possible mechanisms:

photosynthetic, marine algae (Jones, 1974; Carpenter, 1978; Valiela, 1979; Joye, 1994; Currin, 1996; Herbert, 1999) and heterotrophic bacteria (Jones, 1974; Carpenter, 1978; Teal, 1979; Valiela, 1979; Nedwell, 1980; Whiting, 1986; Gandy, 1988; O'Donohue, 1991; Newell, 1992; Joye, 1994; Currin, 1996; Herbert, 1999; Nielsen, 2001; Thomas, 2001; Steppe, 2002; Tyler, 2003). Fixation by algae generally exhibited higher rates in comparison to bacterial fixation. However, fixation by bacteria may be a more important source for fixed N for the local ecosystem.

Algal mats are present in many salt marshes. These mats usually reside in concentrated areas, during the warmer months of the year. Bacteria, on the other hand, exhibit less spatial and temporal variability than the algae, although number densities may decrease during colder months (Nedwell, 1980). Bacterial fixation is therefore able to provide N to a larger area of the marsh than the input via algal fixation. Secondly, many salt marshes are influenced heavily by the tides. Algae exist on the surface of the marsh. The N fixed by algae is subject to exportation from the marsh by the ebbing tide. N fixed by bacteria generally occurs at depth within the sediment, closer to the roots of plants in the marsh. Despite the lower rates of bacterial fixation, the N that is produced is readily available to the higher plants because of the location of the bacteria (Jones, 1974).

Bacterial N_2 fixation has been shown to occur heterotrophically and at significant rates in salt marsh sediments, particularly in microcosms found below the surface of creek banks, in which the presence and activity of sulfate-reducing bacteria were observed (Jones, 1974). The sulfate-reducing bacteria *Desulfovibrio, Clostridium,* and *Bacillus* are also known N_2-fixers and have been measured in high quantities within salt marsh sediments.

Although previous studies measured decreased sulfate reduction rates during the winter, N_2 fixation appeared to be relatively constant in removing most of the N_2 produced during denitrification regardless of the season (Figure 4.2, Figure 4.3, Newton, 2006). Seasonal variability in the onset of manganese-catalyzed nitrification and anammox was observed. Koretsky (2003) reported seasonal trends in dissolved Mn^{2+}, which is produced as a result of Mn reduction. Reduced Mn reduction during the winter would explain the lack of observable Mn-catalyzed nitrification, which appears to stimulate anammox in these salt marsh sediments (Newton, 2006).

The summer incubation data (Figure 4.2 a,b) suggested that some ammonium oxidation pathway leading to the production of N_2 was occurring in the Skidaway Island salt marsh sediments. The previous study by Newton (2006) was conducted within meters of this sampling site and observed Mn-catalyzed nitrification followed by anammox. Production of $^{29}N_2$ or $^{30}N_2$ from amendments using the $^{15}NH_4^+$ tracer, followed by consumption of N_2 would have provided solid evidence for the co-existence of Mn-catalyzed nitrifying, anammox and heterotrophic N_2-fixing bacteria in winter salt marsh sediments. The existence of alternate routes of fixed N removal, based on the Netwon (2006) study, indicated that N was being lost at even greater rates than previously estimated. The co-existence of N_2 fixation with these alternate removal mechanisms would have suggested a balance between the loss and input terms, thus preventing the system from potential N limitation.

4.5.4 Local N Budget

Based on N_2 measurements over the course of the 18-day winter incubation, N_2 fixation

seemingly consumed 90% and 91% of the total amount of $^{29}N_2$ and $^{30}N_2$ produced, respectively. Denitrification is only removing roughly 10% of fixed N from these salt marsh sediments. These results are based on "potential" N_2 fixation experiments using static incubations that may produce sediment conditions unlike those of the natural conditions of the marsh. These results are, however, consistent with a previous N budget estimated for a salt marsh in which the total losses of N exceeded the total inputs by 11% (Valiela, 1979). The authors concluded that internal mechanisms such as denitrification and N_2 fixation are balanced enough to maintain the ecosystem and that anthropogenic effects and eutrophication may change the N content of the system.

4.6 Conclusions

Canonical heterotrophic denitrification appeared to be the only mechanism of fixed N removal in the salt marsh sediments of Skidaway Island during the winter of 2008. Previous findings of the alternate fixed N removal pathway of Mn-catalyzed nitrification followed by anammox were observed during warmer months. It appears that seasonal variations in anaerobic respiration activity may be a contributing factor to the onset of these alternate pathways involving ammonium oxidation.

The average rate of heterotrophic denitrification was calculated to be 0.031 nmol N_2 mL^{-1} wet sed. hr^{-1}. N_2 fixation was measured simultaneously with denitrification using isotope tracer coupled to MIMS techniques. The average N_2 fixation rate was 0.040 nmol N_2 mL^{-1} we sed. hr^{-1}. Static sediment incubations determined that at least 90% of the N removed from the sediment via heterotrophic denitrification was returned via heterotrophic N_2 fixation.

4.7 References

An, S., Gardner, W. S. and Kana, T. (2001) "Simultaneous Measurement of denitrification and nitrogen fixation using isotope pairing with membrane inlet mass spectrometry analysis." *Applied and Environmental Microbiology*. **67**(3), 1171-1178.

Carpenter, E. J., Van Raalte, Charlene D. and Valiela, Ivan. (1978) "Nitrogen fixation by algae in a Massachusetts salt marsh." *Limnology and Oceanography*. **232**, 318-327.

Currin, C. A., Joye, S.B., and Paerl, H.W. (1996) "Diel rates of N_2-fixation and denitrification in a transplanted Spartina Alterniflora marsh: Implications for N-flux dynamics." *Estuarine Coastal and Shelf Science*. **42**, 597-616.

Currin, C. A., Newell, S.Y. and Paerl, H.W. (1995) "The role of standing dead *Spartina alterniflora* and benthic microalgae in salt marsh food webs: considerations based on multiple stable isotope analysis." *Marine Ecology-Progress Series*. **121**, 99-116.

Eriksson, P. G., Svensson, J.M., and Carrer, G.M. (2003) "Temporal changes and spatial variation of soil oxygen consumption, nitrification and denitrification rates in a todal salt marsh of the Lagoon of Venice, Italy." *Estuarine Coastal and Shelf Science*. **58**(4), 861-871.

Fonselius, S., Dyrssen, D. and Yhlen, B. (1999) Determination of hydrogen sulphide. *Methods of Seawater Analysis*. (K. Grasshoff, Kremling, K. and Ehrhardt, M.)**:** 91-100, Wiley-VCH.

Gandy, E. L., and Yoch, Duane C. (1988) "Relationship between nitrogen-fixing sulfate reducers and fermenters in salt marsh sediments and roots of Spartina Alterniflora." *Applied and Environmental Microbiology*. **54**, 2031-2036.

Hammersley, M., and Howes, BL. (2005) "Coupled nitrification-denitrification measured in situ ina Spartina alterniflora marsh with a (NH4+)-N-15 tracer." *Marine Ecology-Progress Series*. **299**, 123-135.

Hansen, H. P., Koroleff, F. (1999) Determination of nutrients. *Methods of Seawater Analysis*. (K. Grasshoff, Kremling, K., and Ehrhardt, M.)**:** 159-228, Wiley-VCH.

Herbert, R. A. (1975) "Heterotrophic nitrogen fixation in shallow estuarine sediments." *Journal of Experimental Marine Biology and Ecology*. **18**, 215-225.

Herbert, R. A. (1999) "Nitrogen cycling in coastal marine ecosystems." *FEMS Microbiology Reviews*. **23**, 563-590.

Hulth, S., Aller, R. C., and Gilbert, F. (1999) "Coupled anoxic nitrification/manganese reduction in marine sediments." *Geochimica et Cosmochimica Acta*. **63**(1), 49-66.

Jones, K. (1974) "Nitrogen fixation in a salt marsh." *The Journal of Ecology*. **622**, 553-565.

Joye, S. B., and Paerl, H.W. (1994) "Nitrogen cycling in microbial mats - Rates and patterns of denitrification and nitrogen fixation." *Marine Biology*. **119**(2), 285-295.

Lovell, C. R., Friez, Michael J., Longshore, John W. and Bagwell, Christopher E. (2001) "Recovery and phylogenetic analysis of *nifH* Sequences from diazotrophic bacteria associated with dead aboveground biomass of *Spartina alterniflora*." *Applied and Environmental Microbiology*. **67**(11), 5308-5314.

Luther, G. W., Sundby, B., Lewis, B. L., Brendel, P. J., and Silverberg, N. (1997) "Interactions of manganese with the nitrogen cycle: Alternative pathways to dinitrogen." *Geochimica et Cosmochimica Acta*. **61**(19), 4043-4052.

Marino, R., Chan, Francis, Howarth, Robert W., Pace, Michael and Likens, Gene. (2002) "Ecological and biogeochemical interactions constrain planktonic nitrogen fixation in estuaries." *Ecosystems*. **5**, 719-725.

Nedwell, D. B., and Abdul Aziz, S. (1980) "Heterotrophic nitrogen fixation in an intertidal saltmarsh sediment." *Estuarine and Coastal Marine Science*. **10**, 699-702.

Newell, S. Y., Hopkinson, C.S. and Scott, L.A. (1992) "Patterns of nitrogenase activity (acetylene reduction) associated with standing, decaying shoots of *Spartina alterniflora*." *Estuarine Coastal and Shelf Science*. **35**, 127-140.

Newton, J. D. (2006). Evidence for manganese-catalyzed nitrogen cycling in salt marsh sediments. School of Earth and Atmospheric Sciences. Atlanta, GA, Georgia Institute of Technology. **Master of Science:** 80.

Nielsen, L. B., Finster, K., Welsh, David T., Donelly, A., Herbert, Rodney A., de Wit, Rutger and Lomstein, Bente Aa. (2001) "Sulphate reduction and nitrogen fixation rates associated with roots, rhozomes and sediments from *Zostera noltii* and *Spartina maritima* meadows." *Environmental Microbiology*. **3**(1), 63-71.

O'Donohue, M. J., Moriarty, D.J.W. and Mac Rae, I.C. (1991) "Nitrogen fixation in sediments and rhizosphere of the seagrass *Zostera capricorni*." *Microbial Ecology*. **22**, 53-64.

Risgaard-Petersen, N., Meyer, Rikke L and Revsbech, Niels P. (2005) "Denitrification and anaerobic ammonium oxidation in sediments: effects of microphytobenthos and NO_3^-." *Aquatic Microbial Ecology*. **20**, 67-76.

Scott, J. T., McCarthy, Mark J., Gardner, Wayne S. and Doyle, Robert D. (2008) "Denitrification, dissimilatory nitrate reduction to ammonium, and nitrogen fixation along a nitrate concentration gradient in a created freshwater wetland." *Biogeochemistry*. **87**, 99-111.

Steppe, T. F., and Paerl, H.W. (2002) "Potential N_2 fixation by sulfate-reducing bacteria in a marine intertidal microbial mat." *Aquatic Microbial Ecology*. **28**, 1-12.

Teal, J. M., Valiela, Ivan and Berlo, Diane. (1979) "Nitrogen fixation by rhizosphere and free-living bacteria in salt marsh sediments." *Limnology and Oceanography*. **24**(1), 126-132.

Thomas, C. R., and Christian, R.R. (2001) "Comparison of nitrogen cycling in salt marsh zones related to sea-level rise." *Marine Ecology-Progress Series*. **221**, 1-16.

Tyler, A. C., Mastronicola, Tracie A. and McGlathery, Karen J. (2003) "Nitrogen fixation and nitrogen limitation of primary production along a natural marsh chronosequence." *Oecologia*. **136**, 431-438.

Valiela, I., and Teal, John. (1979) "The nitrogen budget of a salt marsh ecosystem." *Nature*. **280**, 652-656.

Wang, D., Chen, Zheniou, Wang, Jun, Xu, Shiyuan, Yang, Hongxia, Chen, Hua, Yang, Longyuan, Hu, lingzhen. (2007) "Summer-time denitrification and nitrous oxide exchange in the intertidal zone of the Yangtze Estuary." *Estuarine Coastal and Shelf Science*. **73**(1-2), 45-53.

Whiting, G. J., Gandy, Edwin L. and Yoch, Duane C. (1986) "Tight coupling of root-associated nitrogen fixation and plant photosynthesis in the salt marsh grass Spartina alterniflora and carbon dioxide enhancement of nitrogenase activity." *Applied and Environmental Microbiology*. **521**, 108-113.

CHAPTER 5

CONCLUSIONS

This dissertation provides insights into mechanisms and rates of the major input and loss terms of the local marine N cycle, as well as a technique used to measure both denitrification and N_2 fixation. The results presented represent the conditions of N cycling in Georgia, however inferences into patterns of global N cycling can be made for sedimentary environments similar to those studied in this work. The major findings of this dissertation are summarized below:

5.1 Denitrification in sandy sediments

On the continental shelf of Georgia, canonical heterotrophic denitrification appears to be the dominant pathway for fixed N removal. The results obtained from sediment core incubations using the isotope pairing technique revealed that NH_4^+, which was labeled with ^{15}N-N, was not responsible for the production of N_2. However, sediment cores amended with $^{15}NO_3^-$ showed production of $^{29}N_2$ and $^{30}N_2$ (Figure). This data suggests that canonical heterotrophic denitrification was the sole pathway of fixed N removal. A recent study that examined denitrification in the same location as the study reported in chapter 2 suggested that coupled nitrification-denitrification was the mechanism responsible for fixed N removal. These two studies differed in a number of ways, which may explain the discrepancies between the two findings. The study by Rao et al. (2007) utilized a flow-through incubation technique whereas this study employed static incubations. Although O_2 was present in the cores throughout the 48-hour incubation

period, the use of static incubations prevents the advective flow of O_2 into the sediments, which is a vital component for coupled nitrification-denitrification.

Denitrification was estimated to produce 22.8 – 34.1 μmol N m^{-2} hr^{-1}. These rates were at least two orders of magnitude lower than rates obtained from previous studies of denitrification in fine-grained continental shelf sediments. Rates obtained by Rao et al. were 1.06 – 6.09 x 10^{-6} mmol N cm^{-2} hr^{-1}, which is equivalent to 10.6 – 60.9 μmol N m^{-2} hr^{-1}. In their paper, it is reported that their rates are 10 – 60 times higher than the rates obtained in chapter 2 (Vance-Harris, 2005). Based on the conversions above, their rates are actually very close to those found in chapter 2. Despite the different mechanisms of N_2 production proposed by the two studies, it is clear that SAB sediments produce N_2 within the range reported by Rao et al. (2007), which is inclusive of the range reported here.

New mechanisms and locations of fixed N removal are being discovered, which appears to increase the global rate of N loss from the oceans. The direct measurement of denitrification measured in Georgia shelf sands may help to decrease these estimated rates of fixed N removal. Previous estimates used stoichiometric relationships to calculate denitrification rates (Seitzinger, 1996), which were found to be higher than the rates reported here and by Rao et al (2007). It is clear that improved techniques and higher frequencies of direct measurements may lead to more accurate estimates of denitrification, not necessarily higher estimates of the process.

5.2 The O_2 Effect on N_2 measurements using Membrane Inlet Mass Spectrometry

N_2 and O_2 have been shown to interact within the ion source of the MIMS to create NO^+ ions. These ions interfere with denitrification measurements using MIMS in two ways: N_2 is consumed during NO^+ formation. NO^+ also interferes with measurements of $^{30}N_2$, which is produced during isotope tracer studies, as they have the same m/z of 30. Although this O_2 effect was reported by Eyre et al. (2002), some studies have indicated the O_2 effect was not significant for their particular MIMS. The O_2 effect was observed on the MIMS used in this study. Upon investigation of other MIMS studies, it was determined that the flow rates used by various labs were different. An experiment was performed in which the flow rate was varied and the responding pressure for each flow rate was measured. Applying the ideal gas law and a simple kinetic rate equation, the number densities of N_2 and O^+, as well as NO^+ were determined. Based on the results, the difference in flow rate appears to be indirectly responsible for the O_2 effect in the MIMS. Slower flow rates resulted in lower NO^+ production. However, slower flow rates also influenced the amount of time required to analyze each sample. An optimal flow rate of 1mL/min was determined in order to balance NO^+ formation and sampling time.

5.3 Seasonal variability in N cycling in salt marsh sediments

Heterotrophic N_2 fixation and denitrification were measured simultaneously in Georgia salt marsh sediments during the summer and winter. Anaerobic ammonium oxidation was observed in the summer but was not found to be significant in the winter. The results of the summer incubation are consistent with results from a recent study conducted

within meters of this study, which determined the presence of Mn-catalyzed nitrification and anammox. Previous studies of Georgia salt marshes found significant seasonal variability in the rates of anaerobic respiration, specifically sulfate, iron, and manganese reduction. These results complement the previous studies by suggesting that mechanisms of anaerobic respiration also vary seasonally. Interactions between N and these other elements appear to be significant in controlling N cycling reactions.

N_2 fixation is seldom measured in the winter due to decreased activity by sulfate-reducing bacteria, which have been shown to be the dominant N_2-fixers in salt marsh sediments. Rates of fixation were, therefore, very low. Almost 90% of the N_2 that is produced during denitrification is subsequently fixed. These results agree with previous studies of N_2 fixation in salt marshes that suggest that denitrification and N_2 fixation are responsible for the internal cycling of N and balance one another to prevent large net fluxes of fixed N into and out of the salt marsh.

5.4 Future Research Directions

The goal of constraining the global marine N budget is quite lofty, but the contributions of this work, although small in scope and scale, will certainly provide the direct measurements of denitrification and N_2 fixation in Georgia continental shelf and salt marsh sediments, which are absolutely necessary to obtaining accurate rates for input into global models. Also, the MIMS techniques used and developed in this dissertation will serve as a model for direct measurements in other locations where rates of denitrification and N_2 fixation are relatively low.

It is becoming more important that the global marine N budget be constrained as recent studies indicate that there may be an overabundance of fixed N entering the oceans in the near future because of anthropogenic activities (Galloway, 2006). The techniques used in chapter 4 can now be used to accurately measure N_2 in environments containing a wider range of O_2 concentrations. Simultaneous measurements of denitrification and N_2 fixation in salt marsh sediments using MIMS should be conducted in other locations within the salt marsh, such as the high marsh and creek bank, in order to determine spatial variability. The results of chapter 2 coupled with a deeper understanding of N cycling in the Georgia marsh would aid in the determination of an annual N budget for the entire Georgia coastal system.